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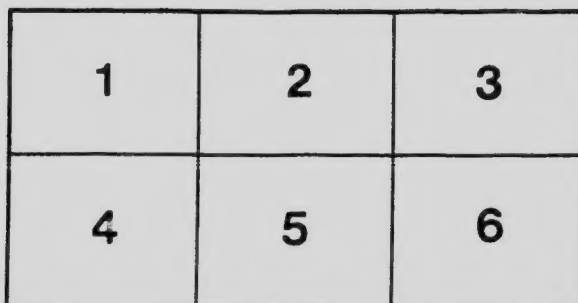
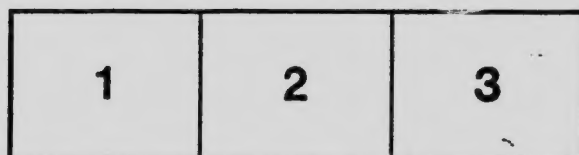
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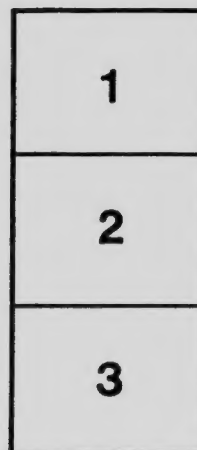
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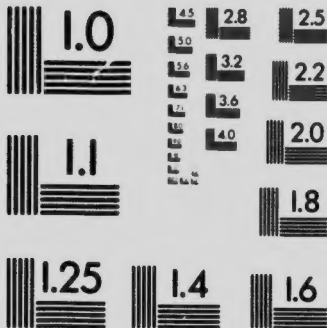
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DOURINE IN CANADA

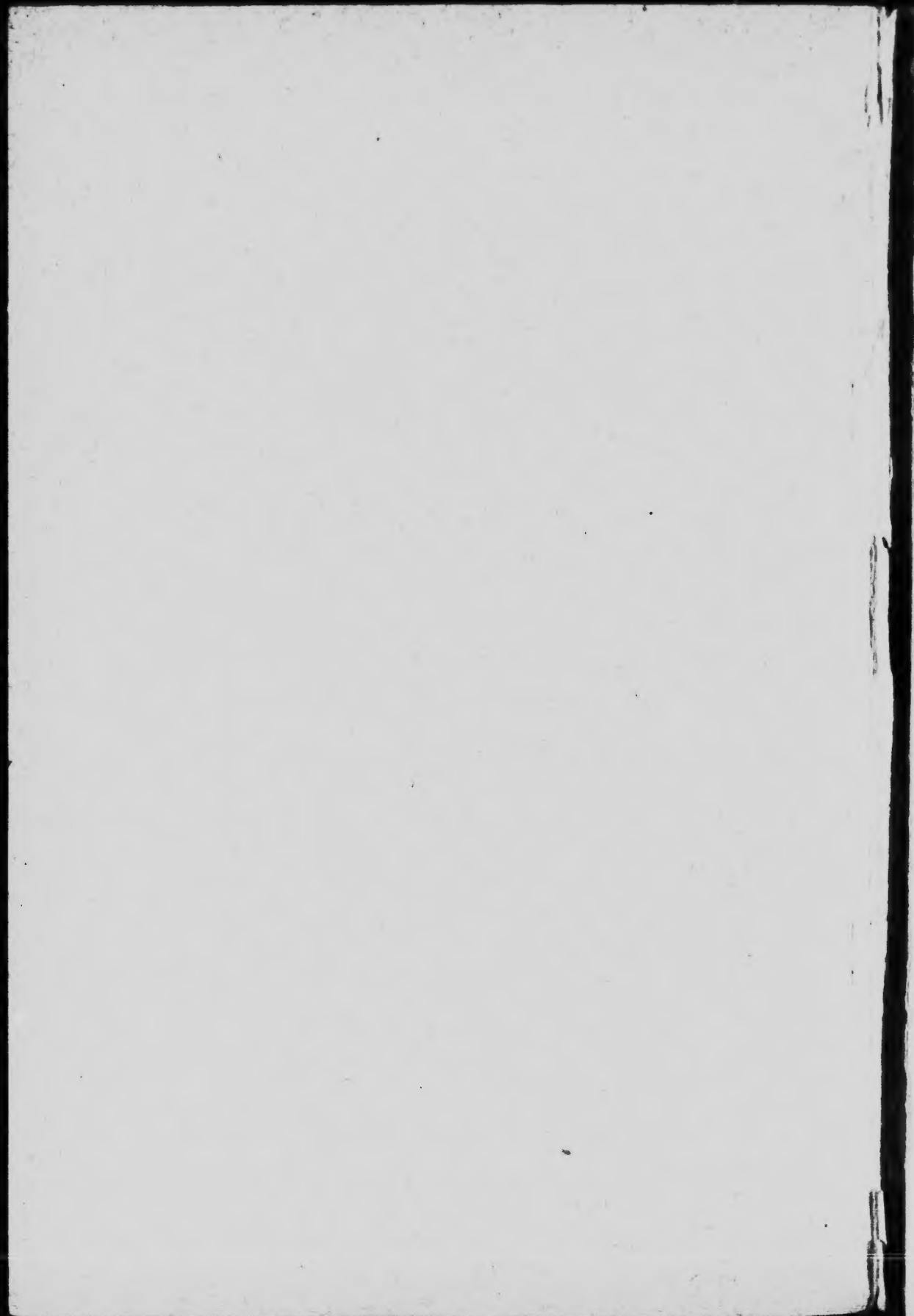
1904-1920

HISTORY, RESEARCH AND SUPPRESSION

BY
E. A. WATSON, V.S., (Capt. C.A.V.C.)
CHIEF ANIMAL PATHOLOGIST

OTTAWA
THOMAS MULVEY
PRINTED AT THE KING'S MOST EXCELLENT MAJESTY
1920

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DOURINE IN CANADA

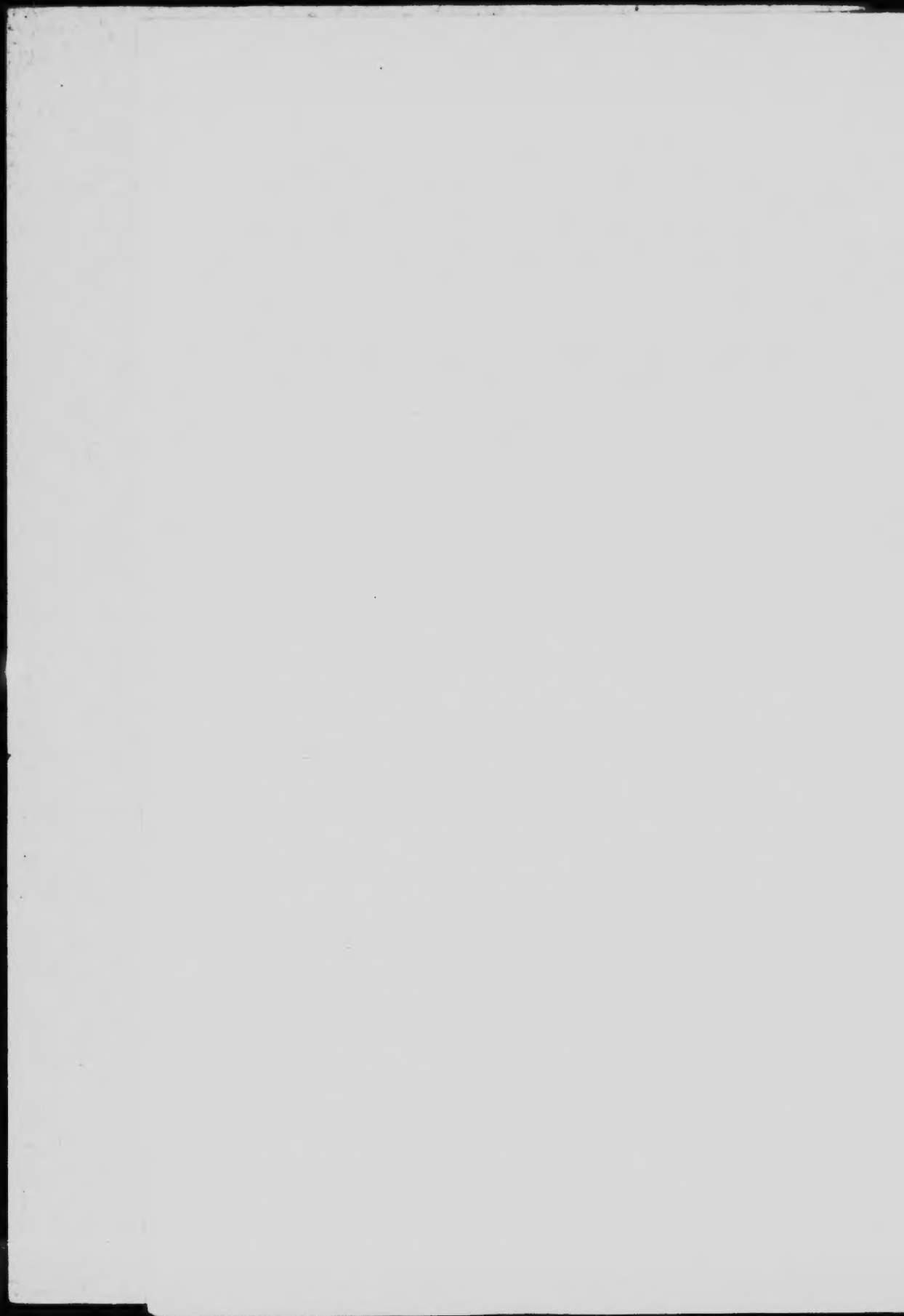
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INTRODUCTION.

Canadian experience with dourine extends over a period of fifteen years and covers the study and suppression of a rare and serious disease hitherto unknown in Canada.

Although the disease is one of the oldest recorded in veterinary literature and has been known to the Arabs and in certain countries of Asia and Africa for centuries, it is only within the past twenty-five years that the nature of it has come to be recognized and understood. The blood parasite *Trypanosoma equiperdum*, seen for the first time by Rouget, in 1894, was for a long time contested by leading veterinarians of Europe as being the specific cause of dourine, and the question was still in dispute ten years later when the disease made its first appearance in Canada.

It was then recognized by Inspector Burnett (1904), and immediately afterwards by Dr. Rutherford, then Veterinary Director General, who, as a result of his investigation and in the absence of positive proof, held to his conclusions and adopted a policy the wisdom of which was borne out by subsequent events. The long search for the specific cause of the disease and its discovery; the experimental work patiently and labouriously pursued year after year; the determination of specific serum methods of diagnosis, by the aid of which the disease was eventually suppressed; and the difficulties met with and overcome both by the veterinary inspectors in charge of the field work and by the pathologists in the laboratory—furnish an example of persistent and unrelenting effort and success achieved in an important problem of veterinary research and sanitary science.

This report includes a review and summary of papers and records published in the Annual Reports of the Veterinary Director General for the years 1904 to 1919, a general description of the disease, and a number of appendices.

It is presented by permission of Dr. F. Torrance who for the past seven years has directed the work of suppressing dourine and has brought it to success.

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HISTORY, RESEARCH AND SUPPRESSION.

Review and Summary.

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The disease was first observed in the province of Alberta, in the Lethbridge district, in the early spring of the year 1904. From the inquiries made in the neighbourhood it appeared probable that the disease had been introduced several years previously. Though the origin was never exactly located there is good reason for attributing it to animals brought in from the Western United States in which, among range animals, the disease was known to exist from time to time.

In March, 1904, Inspector Burnett, Chief Veterinary Officer of the Royal North West Mounted Police, reported the existence of dourine (*Maladie du Coit*) in a stallion and several mares, located near Lethbridge, Alberta. Dr. Rutherford, Veterinary Director General, Dr. Hargrave, of Medicine Hat, Dr. Davison, of the United States Bureau of Animal Industry, and other veterinarians examined the affected animals and agreed upon the diagnosis.

A quarantine station of 1,800 acres of fenced land was thereupon established near Lethbridge, where all suspected animals were gathered in and held under observation. Under the Animal Contagious Diseases Act, 1903, regulations relating to this disease were formulated and brought into force (Appendix No. 5). Provision was also made for the slaughter of infected animals and the payment of reasonable compensation to the owners.

The quarantined animals were re-examined after an interval of several months and when it was expected that acute or well-marked symptoms of the disease would have developed. Dr. Rutherford reported that instead of finding an aggravation of symptoms and a progressive state of disease, a surprise was encountered in that no well-marked case was presented, that many of the affected animals had improved in a marked degree, and that there was not among them any one case sufficiently pronounced to warrant an order for its destruction.

Doubts began to arise as to whether the disease was actually dourine or some other. Many examinations of blood and of the body fluids obtained from affected animals were made in the field, and specimens were sent to various laboratories, but without revealing any trace of the presence of the parasite or any definite information as to the nature of the disease. Dr. Rutherford drew attention to the fact that dourine as an indigenous disease in Asia and in Africa had become more irregular and uncertain in its course and manifestations in Europe and in America, and, probably, still further modified under the peculiar climatic conditions of the northwest provinces of Canada. The decision was made not to immediately adopt a policy of slaughter, but to hold the suspected animals for a further period in quarantine until more satisfactory evidence was obtainable, and also for the purpose of acquiring as much information as possible as to the behaviour of the disease in this country.

In May, 1905, the animals were again examined by Dr. Rutherford and accompanying veterinary officers. In some cases the disease was found to have made progress, in others there was little change from conditions noted on previous occasions. One hundred and sixteen horses were destroyed; a considerable number held over for further observation. Special officers were detailed to deal with the disease and were authorized to order the slaughter of any clearly marked cases coming under their notice.

In September, 1905, it was decided to slaughter all the animals at the quarantine station excepting a few which had been under observation for upwards of twelve months and appeared to be healthy. It was then arranged to utilize the existing

quarantine station for experimental work in connection with dourine, and Dr. Hadwen, an inspector at Nelson, B.C., was transferred to Lethbridge and placed in charge, with a number of condemned animals at his disposal. Three infected animals were sent to the Biological Laboratory, Ottawa, for study and experiments.

Investigations were continued in the field and in the laboratories during the winter of 1905-06 and the following summer. The disease was found to exist in various parts of southern Alberta, and by October 31, 1906, 412 animals had been destroyed.

At the Lethbridge quarantine station breeding experiments were conducted with the object of ascertaining whether apparently recovered mares would bear healthy offspring or would remain sterile. Diseased and healthy stallions were employed in these experiments. Several slightly affected mares were spayed in the hope that such cases could be spared destruction and usefully employed for work purposes only. The search for the dourine parasite *Trypanosoma equiperdum* was kept up continuously both at Lethbridge and at Ottawa. Microscopical examinations were made of great numbers of specimens; dogs and other laboratory animals were inoculated with blood and material obtained from diseased tissues, but all were negative in result and every attempt to detect the parasite met with failure.

At the Biological Laboratory, Ottawa, Higgins and Watson had made a cytological study of the blood of dourined horses comparatively with the blood of normal horses and in certain diseases. It was found that the leucocyte formula in dourine differed from that in normal horses and in the other diseases studied, and was of some diagnostic value. A pathogenic trypanosome, *Tr. gambiense*, was maintained and studied in a series of laboratory animals, mainly with the idea of acquiring a knowledge of the habits and characteristics of a blood-haunting protozoan parasite closely allied to that of dourine. In October, 1906, Watson took over the investigation of dourine and removed to Lethbridge. In November and December of that year he discovered trypanosomes in the blood of native "cotton-tail" rabbits and in a species of northern deer-mouse caught on the quarantine grounds at Lethbridge. These findings created much interest at the time, being the first record of trypanosomes found in mammalian blood in Canada.

The important discovery of *Tr. equiperdum* was made in February, 1907, by Watson, in a dourined mare in which the disease had been recognized by Inspector Gallivan. The reproduction of the disease by inoculations of this parasite into healthy horses, and the recovery of the parasites from the typical lesions produced, removed all doubt as to whether the dourine of America was a trypanosome disease or not, and clearly established its identity with the dourine of Asia and Africa and of European countries. However, in Africa and Asia, where the disease exists in its most severe form, no great difficulty seems to have been encountered in detecting the parasite or in diagnosing the disease by its clinical manifestations. In Europe, on the contrary, the search for the trypanosome was for many years unsuccessful, the course of the disease was found to be irregular and uncertain, and diagnosis, as a rule, had to be based upon clinical symptoms, which, as often as not, were too vague and elusive to permit of satisfactory conclusions. In America it was already evident that the disease occurred in a still more insidious form and that frequently in the early stages of infection, which might continue for very lengthy periods, neither a characteristic symptom nor a warning sign of the disease was presented. Further, and far from being an invariably fatal disease, many infected mares retained or recovered apparent health, though suspected of being capable of transmitting the disease to the covering stallion. The better bred and especially the pure-bred imported animals of high commercial value were usually the most severely affected and the first to succumb. Prairie-bred animals of the native pony and broncho types were best able to resist or tolerate dourine infection and to lend themselves as carriers of it, and in the absence of any reliable means of recognizing them as such, constituted a frequent and dangerous source of infection. At this time large herds of horses lived and bred on the open ranges where natural conditions all favoured the spread of the disease.

The horse industry was thus faced with a serious menace and the Veterinary Department with the formidable task of overcoming it. The prospect of eradication in face of the apparently insurmountable difficulties in diagnosis did not look encouraging. It was decided to carry on research, to study the disease from its various aspects and, if possible, devise a specific or accurate method of diagnosis which would enable the department to deal with dourine in an efficient and satisfactory manner and to finally suppress it in its entirety.

During the next five years, 1907-12, cases of dourine were discovered in a number of the breeding districts in the provinces of Alberta and Saskatchewan and reported on by Inspectors Gallivan and W. L. Hawke. The procedure when an animal was found affected was to secure the fullest possible history of the case, to trace all animals which had been in contact by breeding and to quarantine sick animals for an indefinite period, forbidding them to be bred, sold or removed without a special license. Periodical examinations were made by the inspectors in charge, and as soon as a clear case of disease had developed the animal was destroyed. But it happened quite commonly that no definite symptoms could be detected until well on into the second year of the disease, and that in consequence horse-breeding among such groups of animals, often several hundred in numbers, had to be prohibited for two and sometimes three years in succession. Even then there was no certainty that some latent carrier did not remain undetected. In fact, as became known later on, in any large outbreak of dourine and especially among prairie-bred animals, it is practically certain that such carriers do remain and can only be detected by specific methods of diagnosis. Although no better measures of control could be devised under the existing circumstances, the prolonged quarantine for observation purposes, the periodic examination by the veterinary inspectors and the maintenance of large breeding establishments or herds of brood mares which must not be used for breeding purposes, proved costly, onerous and unsatisfactory. These five years (1907-12), saw a great amount of research and experimental work accomplished at the Quarantine and Research Laboratory, Lethbridge. Besides the original trypanosome strain several others were obtained from different sources of dourine outbreaks. Parallel series of experiments were made with the different strains in a close study of the pathogenicity, life-history and variations in the virulence of *Tr. equiperdum*. The strains were maintained in horses: the evolution of the disease in its various phases was followed from year to year and many observations and experiments were made upon the survivors. Mares which had spontaneously recovered, some from a natural and others from an experimental infection, were successfully bred and raised healthy offspring year after year. It was found that these mares resisted reinfection and that the serum of such animals had immunizing properties. The experimental treatment of dourine was investigated both from a preventive and a curative point of view, immunizing sera and special drugs recommended in other trypanosome diseases, e.g., atoxyl and arsenophenylglycin, were employed and reported upon. The chief objective, that of determining a precise method of diagnosis, was always kept in view, and to that end the study of the serum properties of dourined animals was a continuous one. Serum reactions and serum methods of diagnosis were worked out and included a serum-globulin test, a trypanosome agglutination test and a precipitin test. Of these, the agglutination test was the more satisfactory but did not meet all requirements.

The winter of 1911-12 was spent by Watson in a study of trypanosome diseases in European laboratories. A German strain of dourine (Beschläuseuche) and an African strain were obtained from the Imperial Health Institute, Berlin, and brought to Canada for comparative work. Good progress in the serum diagnosis of dourine was made during the year 1912. The Complement-fixation or Complement-deviation test,* after several modifications and improvements in technique had been made, was found to provide a specific, precise and uniform method of testing. As a means of diagnosis it answered all requirements and came into general use during the year 1913. Towards

*See Appendix No. 2, pages 16, for explanation and description of the Complement-fixation test for dourine.

the end of that year a recurrence of the disease was reported in the southern districts of Alberta. The field investigations showed it to be of very wide extent and to cover the old centres of infection of the first known outbreaks occurring six to eight years previously. On one company's ranch 2,718 horses were involved, including a number of imported stallions and mares and several hundred animals of high grades and value. Blood was collected from every animal and sent to the laboratory. As a result of the first test over 400 animals were pronounced infected. Some scepticism was at first evinced by the owners as animals, apparently in perfect health, were condemned on the strength of a positive serum reaction. But as many of these animals began to show symptoms and when several had died, all opposition vanished and the owners were willing enough to have the animals slaughtered and compensated for, and to fully co-operate in stamping out the disease.

The Complement-fixation test has since been employed wherever the disease has made an appearance in this country. It has proved of inestimable value as the main factor in the diagnosis and suppression of the disease, enabling an early and definite decision to be made—just as soon as blood can be collected and sent to the laboratory. Instead of holding suspected animals in quarantine for indefinite periods extending into years it has sufficed to apply the test and, after a short interval and to eliminate any doubtful reactors a retest. It was most fortunate that this test was available when the recurrence and wide extent of the disease became known in the years 1913 to 1916. During this period every effort was made to cover the whole field of the disease, and it was then that the heaviest toll was paid in slaughter and in compensation. The large Indian reservations situated within the infected area of southern Alberta were for long suspected of harbouring dourine. It was rarely possible to detect a case among the native ponies themselves but cases were frequently found among the better grades of animals of the neighbouring ranchers which from time to time strayed on to the Indian ranges. A serum test of these Indian herds indicated many cases of hidden infection and of dourine carriers. This was not surprising as the constant intermingling of herds, the Indian habit of letting young stallions go uncastrated until three or four years of age, and promiscuous breeding had given every facility for the scattering of infection. To drain out this remaining source the herds were gathered up and tested repeatedly at intervals of six months to one year, and the animals whose serum reacted positively in the complement fixation test were destroyed.

In connection with the serum-diagnosis of dourine over 40,000 laboratory tests were made during the period 1912-20.

Since the disease first came under departmental action and up to present date (1904 to 1920), 1,933 horses have been destroyed.

The figures year by year are to be found in the table of dourine statistics given below.

At the present day dourine appears to have been totally suppressed in Canada. If further outbreaks should occur or the disease be reintroduced it should never have an opportunity of gaining much headway now that there are the means of detecting it either in its initial or active stages or in its latent and hidden forms, and thus dealing with it promptly and efficiently.

Finally, the testing of all stallions imported into the country and stallions that are travelled through the country for breeding purposes would prevent the reintroduction of the disease and afford an adequate protection to the horse industry.

DOURINE STATISTICS (1904-20).

Year.	Horses destroyed.	Value.	Compensation
		\$	\$
1904-05.....	292	24,045 00	16,029 94
1905-06.....	120	10,210 00	6,806 48
1906-07.....	167	15,505 00	10,336 44
1907-08.....	49	5,175 00	3,449 92
1908-09.....	28	3,760 00	2,506 54
1909-10.....	37	5,130 00	3,419 98
1910-11.....	40	4,960 00	3,306 60
1911-12.....	18	2,610 00	1,739 99
1912-13.....	18	3,145 00	2,096 00
1913-14.....	471	73,115 00	48,743 33
1914-15.....	394	48,931 00	32,080 66
1915-16.....	228	26,085 00	17,389 65
1916-17.....	48	4,924 00	3,222 63
1917-18.....	16	2,011 00	1,340 66
1918-19.....	5	392 00	261 33
1919-20.....	2	105 00	70 00
	1,933	230,103 00	152,800 15

NOTE.—During the present year commencing April 1st, up to the time of going to press, November, 1920, not a single case of dourine has been reported. The final test of the last remaining herd under suspicion—about one thousand mares and stallions running upon an Indian Reservation—has been concluded, all the reactions being negative.

DOURINE.

THE NATURAL DISEASE.

As it occurs in Canada dourine infection is directly communicable from the diseased to the healthy animal by either the horse or the ass through the act of sexual union and in no other way. It is caused by a protozoan parasite named *Trypanosoma equiperdum* and is the only known disease of trypanosomian origin that occurs in Canada. Natural dourine is very rarely, if ever, observed in an acute form. Starting from an infective coitus, at some point in the genital organs the disease takes an irregular and uncertain course but is essentially chronic and intermittent. Determining factors in its evolution are (1) the degree of virulence of the trypanosome strain, (2) the degree of resistance or of susceptibility of different breeds of horses, and (3) physical and functional conditions and disturbances dependent upon climate and seasonal changes, breeding, exhausting work, and stabling or living a natural life on the open ranges or pastures. As to virulence, Laveran and Mesnil have well stated that "the trypanosome of dourine constitutes one of the best examples of variations in the virulence of the same species following its origin and genealogy." The higher breeds of horses show the greater susceptibility. In the thoroughbred the disease is apt to make quick progress and manifest itself mainly in derangements of the central nervous system. In the heavier and sluggish breeds, such as the "Clydesdale," the disease is more halting and is marked mainly by oedema and disturbances of the circulatory system. Native pony breeds are the most resistant and frequently serve as carriers and reservoirs of the virus for a duration of years. Donkeys are also well able to tolerate infection and to become dangerous carriers. Climatic changes and extremes of temperature may cause either an arrest or an advance in the progress of the disease, cold retarding and heat favouring it. Thus in Canada it often lies dormant through the winter months, becoming suddenly and increasingly active during the first hot spell of weather in June or July. Under natural conditions on the open range or at pasture more resistance is shown to the disease than in stable-kept animals. Breeding, heavy work and physical exhaustion favour the disease, especially in the stallion. Mares that have become impregnated at the time of the infective coitus occasionally abort, but more often they carry their young to full term. It has been noted again and again that pregnant infected mares have remained apparently healthy up to the time of parturition and that shortly after and about the time of the first oestrus have changed to a state of obvious disease and rapid breakdown in which marked symptoms of dourine were presented. In short, while all horses are more or less susceptible to dourine, the disease itself, like most chronic infections, is susceptible to various modifications and interruptions.

Infection.—Although infection is passed from the diseased to the healthy during the act of copulation, and in that manner alone, it does not follow that transmission is always effected at such time of contact. A stallion, for example, may infect all the mares he covers during one week and none during the next. The contact of the two mucous surfaces, the diseased and the healthy, provided the dourine parasite is present at the time, is the only necessary condition for transmission. The trypanosome is quite capable of passing through an intact and mucous membrane and no wound or abrasion is needed for the virus to gain an entry to the tissues. But there are periods in the course of the disease when the parasite is absent from the genital

mucosa and lies dormant in the tissues. These may be called "non-infective" periods and may have a duration of weeks or of months; they are most apt to occur in the later stages and, consequently, animals in the earlier stages of infection are usually the most active propagators, while those which enjoy a temporary or partial immunity become the carriers.

Period of Incubation.—The interval from the time of the infective coitus to the first appearance of symptoms or of visible changes varies and averages between two weeks and three months. It may be even longer and sometimes it happens that no symptoms can be recognized until the first winter has passed and the second spring or summer arrives. From an immunological viewpoint and as a period of sensitization, the incubation interval is less than one month, for specific serum reactions can be obtained within that time and usually in from ten to twenty days after infection.

Evolution of the Disease.—Commencing as a local infection dourine habitually takes a chronic, intermittent and irregular course both in its local and general evolution. With trypanosome strains of low virulence and in animals of strong resistance the infection may be arrested locally and held in check for an indefinite period and, in some cases appears to terminate there. In other cases, and such are in the majority, infection is both local and general, that is to say, it predominates locally, in the genital organs, throughout the course of the disease but, from time to time, finds its way to distant organs and tissues. In a third type of case the opposite holds good, that is, the genital organs acquire a partial or temporary immunity while the disease is progressing in other directions, and especially in its attack upon the central nervous system.

In most accounts of dourine the evolution of the disease is divided into three well-defined periods or stages to which certain symptoms are ascribed, thus: (1) *Primary*—oedema, tumefactions and changes in the genital organs; (2) *Secondary*—plaques and skin eruptions; (3) *Tertiary*—paralyses, anaemia and cachexia. This arrangement is convenient for descriptive purposes but is arbitrary and rather apt to mislead an inexperienced observer of the disease in his conclusions or diagnosis based upon the clinical history and evidence. Actually, the disease operates in a succession of attacks, each attack being followed by a period of tolerance or temporary immunity of very uncertain duration. One attack may follow another so closely that symptoms overlap or are never altogether absent. On the other hand, the attacks may be so far apart that in the long intervals between them there is no visible sign of the disease. Unless such an interval has continued for at least two years without any relapse it would be hazardous to assume that the animal had permanently recovered. As for the grouping and sequence of symptoms it should be remembered that symptoms of all three groups may be coexistent; or, that symptoms of one group may appear simultaneously with those of another, and that "primary" symptoms, or those categorized as such, may reappear after the first, second, third or any later attack, and, similarly, that those of the "secondary" or "tertiary" groups may recur repeatedly and irrespectively of the order or sequence indicated.

In short, the disease is marked by stages of exacerbation, tolerance and relapse, any of which may be of short or of long duration. They may occur but once or may be repeated over and over again before the final stage either of recovery or of decline. The symptoms most frequently noted are fever, tumefactions and local oedemas of the genital parts and mammary glands, oedematous eruptions of the skin, knuckling of joints, and inco-ordinate movement, especially in the hind limbs, facial paralyses, ocular lesions, anaemia and emaciation. The one true pathognomic symptom is the oedematous patch or "plaque"; but, unfortunately, it is a rare symptom and can only be observed in a comparatively few cases. In the earlier stages of infection fugitive oedemas of the genital organs and paroxysms of fever are the rule, thus:—

In a stallion the first symptom is a slight oedema of the sheath, often accompanied by a slight swelling of the glans penis. The mucous membrane at the urethral

exit may show a bulging, infiltrated appearance and may even protrude from the opening. These first oedemas tend to disappear within a few days. After an interval, usually fifteen to twenty days, the oedema returns and is apt to be more pronounced. It may involve a portion of the scrotum and fluctuates as a cold and painless swelling. As a rule there are well marked stages of augmentation and diminution with varying intervals between. The oedema advances in a succession of waves and during each recess an increasing extent of permanently thickened and indurated tissues can be noted. The penis may be in a state of paraphymosis, temporary or more or less permanent. In stallions of the heavy sluggish breeds the oedema may extend over the whole of the floor of the abdomen and reach along the chest wall to the brisket, dominating the clinical picture throughout the disease. In the lighter breeds, and especially in the more active and highly-strung running horses, this symptom may be slight and quite transient.

In a mare the first symptom is a patch of slightly swollen mucous membrane of the vulva or vagina and is closely followed by tumefactions of the vulva. Sometimes only one labium is involved, giving a distorted appearance to the vulva. The oedema vanishes and returns, the intervals becoming more and more irregular. The outer black skin of the vulva, and sometimes of the anus, may show irregular shaped patches of depigmentation. The patches usually extend outwards from the inner borders of the labia, at first appearing pink and then becoming white. They are of uncertain duration; in some cases they persist or remain as permanent leucodermic patches, in others they return to a normal appearance within six months to one year. At this stage the vaginal mucosa may show raised and thickened, semi-transparent patches of glassy or straw-coloured appearance or somewhat mottled. Folds of swollen membrane may protrude into the vulva opening; the clitoris is often swollen and erect. Frequently there are spasmodic attempts to urinate, prolonged periods of oestrus and increased sexual desires. At such times there is an excess of vaginal mucous mixed with a serous exudate and occasionally tinged with blood. In the later stages of the disease, and especially when an infected pregnant mare has aborted or foaled, there may be a chronic vesico-uterine discharge. In some mares the genital symptoms may be so slight as to escape observation. After infection has existed for six months or longer, and when it may be impossible to detect any abnormal condition of the vagina or vulva, it is not uncommon to find a slight oedema of the udder or of the skin and underlying tissues just anterior to the udder. Less commonly there may be noted a oedema about the anus, or one or more patches between the vulva and the udder, or upon the inner surfaces of the thighs. Such symptoms, slight and vague as they may seem to be, are important, for it is in these serosities that the trypanosomes of dourine are most likely to be found by patient searching.

The true "plaque," or "dollar spot," differs somewhat in appearance from the oedematous patches already mentioned. The "plaque" has been aptly described as a thin disc of metal slipped under the skin. As such it may appear upon any part of the body surface, but seems to favour the sides covering the ribs. In Canada the characteristic symptom of dourine can rarely be observed. In many animals held under continuous veterinary observation, and in which this symptom was especially watched for month after month, in winter and in summer, a plaque was never known to have occurred. Others have exhibited plaques in the first, second and in the third years of the disease. In the most of cases the plaque has occurred singly and at wide intervals of time; less often, two, or even three, plaques were observed at the one time; and, in some rare cases, a rapid succession or "crop" of plaques during one brief period in the course of the disease. The average duration of a single plaque is four to five days. Rarely does the eruption and absorption of the swelling occupy less than three days or more than ten days. The average size is one and a half to two inches in diameter and one-half inch in thickness or elevation. The site of a plaque, especially in animals with a smooth and glossy coat, is often made conspicuous by the staring or bristling appearance of the hair at that point. During the eruptive stage of the swelling and in the serous fluid the trypanosome of dourine can always be found.

Fever, distinctly of the intermittent or remittent type, may occur during any stage of the disease. It is more regular and pronounced during the early stages and before any marked tolerance or immunity has been acquired. A typical paroxysm has an average duration of five to six days, the maximum temperature, usually 104 to 106° F., being reached about the third or fourth day. The intermission ranges between twenty-one and twenty-eight days. These febrile paroxysms are closely associated with trypanosome cycles of development. A renewal of genital symptoms, the eruption of plaques, or a recurrence of oedema on some part of the animal body can usually be detected during or just preceding the rise in temperature. As the disease advances the febrile conditions become more variable and inconstant. In the later stages a subfebrile temperature of about 102° F. may continue for weeks or months. Very little depression or constitutional disturbance accompanies the fever, even during the severer paroxysms, and the appetite is maintained throughout.

The nervous system is subject to severe disorders and disturbance, indicated by inco-ordinate locomotory action and paraplegic conditions affecting the hind limbs, lips, nostrils, ear and throat. The animal shows a wabbling gait and sways from side to side. There is knuckling over at the fetlock joints, and, in unshod animals, the toes of the hind hoofs wear down from dragging and shuffling the feet. The animal is prone to trip over small obstacles and there is frequently a spasmodic contraction and relaxation of the muscles of the hind legs. Facial paralysis is nearly always unilateral. The ear, upper eyelid, nostril and underlip of one side of the head may be affected at the same time or only one of these appendages. The nervous symptoms are not as fugitive in character as the symptoms of the other groups. They tend to persist or to become aggravated independently of other symptoms, and, as a rule, are the more pronounced in the absence of other symptoms.

Ocular symptoms are not constant and occur in only a small percentage of cases. They comprise photophobia, lachrymation, keratitis, corneal opacities and changes in the interior of the eye. Stallions seem more prone to ocular troubles than do mares. It is of interest to note that on more than one occasion a corneal opacity was the first symptom observed in animals that came under suspicion of dourine infection.

The duration of the disease averages between one and two years. Rarely is it under six months; sometimes it extends to three years and occasionally to five years. As a rule, if an animal survives the third year of infection a permanent recovery of health follows. In such cases specific infection and immunity reactions are obtained with the complement-fixation test for an indefinite period and even up to 10 years.

Termination and Mortality.—In cases advancing towards a fatal termination the decline is usually slow and gradual. The appetite remains good almost to the end and often appears excessive. There is a progressive anaemia and emaciation; the animal has a most dejected appearance and continues a miserable existence until finally, from exhaustion and paraplegia, it is down and unable to rise, and, unless destroyed, suffers a lingering death.

In stallions the disease would appear to be fatal without exception; but in mares the evolution of the disease is slower and more irregular and uncertain in its result. Certainly, a large number of mares would make a natural recovery. The mortality depends upon the factors of virulence of the pathogenic agent and upon the resistance of the breed of animal infected. In the prairie-bred, native animal, the mortality is low; in pure-bred, imported animals it is high. Among the naturally infected animals, representative of different types and breeds, at the dourine research station, the mortality has not exceeded 50 per cent.

Pathological Anatomy.—The end stages of dourine are usually marked by a progressive emaciation and anaemia and in many cases, and more particularly in stallions, by oedematous swellings extending along the lower surfaces of the body. The oedema is sometimes of enormous extent and the swollen tissues in the region of the scrotum and under the abdomen may be up to eight inches and more in thickness.

At the autopsy one finds more or less gelatinous exudation under the skin. In the stallion the scrotum, sheath and testicular coverings are thickened and infiltrated and in some cases the testicles are embedded in a tough mass of new sclerotic tissue in which they are scarcely recognizable. In the mare the vulva, vaginal mucosa, uterus, bladder and mammary glands may show thickening and gelatinous infiltration. The lymphatic glands, particularly the abdominal, are hypertrophied and softened and sometimes hæmorrhagic.

Further changes similar in character to those found in other chronic, wasting disease and anaemia may be noted, viz., gelatinous infiltration, serous transudation from the pleura, peritoneum and pericardium, and fibrinous deposits, adhesions and new growth on the liver, spleen, diaphragm and thoracic organs. Cystitis, pyelitis, nephritis, and bone marrow changes may also be noted. The spleen is not enlarged and may be under weight. The liver may be engorged with blood.

The principal changes are to be found in the nervous system, and are most marked in the lumbar and sacral regions of the spinal cord. In animals in which paralytic conditions in the hind limbs have been severe and of long duration the cord shows softened, pulpy and discoloured areas. Histological examination shows degeneration of the nerve fibres of the posterior columns, and a cellular infiltration, degeneration and atrophy in the extraspinal nerve trunks, particularly of the hind limbs. In accordance with these findings Marek names the disease *Polyneuritis infectiosa*.

APPENDIX No. 1.

DIAGNOSIS.

Symptomatic diagnosis.—The type of oedema affecting the genital organs, the eruption of the characteristic "plaque" and the peculiar nervous symptoms and inco-ordinate locomotion in connection with the hind limbs may suffice for a diagnosis. But in the early stages of the disease and in latent cases it is difficult or impossible to detect any such symptoms or to recognize them with any certainty. The history of the case or cases suspected and the examination of the contact animals is important and not to be overlooked. Where it may be impossible to come to a conclusion in the case of a single animal, the symptoms presented in a group of animals may clear away all doubt. If it is found that only stallions and brood mares are affected there are but two covering diseases to consider, namely, dourine and coital exanthema. The latter is the disease most commonly confused with dourine owing to a misconception of the relative importance of ulcers and depigmented spots in these diseases. In coital exanthema the ulcers, preceded by vesicles and followed frequently by depigmented spots are characteristic lesions and the basis of a symptomatic diagnosis; in dourine they are not and have very little importance. It has been said and repeated in many descriptions of these diseases that the depigmentation in the former is transient and in the latter permanent. I have found the opposite conditions to prevail. With regard to ulceration of the genital organs in horses it is extremely doubtful if such lesions ever occur as a direct result of dourine or any other trypanosome infection. I have never observed an ulcer in any case of experimental infection in horses. Ulcers have occasionally been noted in naturally infected mares, usually in aged animals, and in stallions, and also in normal mares and stallions. In appearance the lesions are quite similar in the dourined and in the healthy animals. In mares they are usually situated near the edges of the vulva and in stallions on the inverted portion of the penile sheath. Microscopical examination of deep scrapings frequently show spirochaetae infection.

Detection of the parasite.—In the naturally infected animal the trypanosome appears and multiplies in the serosities of local oedemas. It is futile to search for it in the blood. As soon as a plaque is signalled or an oedematous patch in the region of the vulva, anus or mammary gland is noted, the search should commence. The skin over the patch should be washed, shaved and dried. Two or three punctures are then made with a fine needle and if a clear or only faintly tinged serum exudes on pressure wet cover-glass preparations and stained smears are examined. A very long and thorough search should be made before pronouncing a negative result. Sometimes there are one or several trypanosomes present in each microscopic field but this is a rare finding. Sometimes only one or two organisms are to be found in the whole of the specimen examined. The object in puncturing the swelling with a fine needle is to avoid the blood vessels; the more the serous fluid becomes diluted with blood the less the chance of detecting the trypanosome. As a rule the parasites are present in the serous contents of the oedema only for a few days though the swelling itself may persist for several days after parasites have disappeared from it. It may accordingly be necessary to examine a number of oedemas before the parasite can be detected.

In searching for the parasite in the vaginal tract the most oedematous looking patch of mucous membrane is slightly scarified and specimens of the exuding fluids are prepared for examination. A previous irrigation of the vagina with warm salt solution secures fresher specimens, richer in trypanosomes and facilitates the search.

The trypanosome is very rarely to be found in the massive oedema that spreads along the under surface of the abdomen nor in the oedema of the scrotum.

In horses the parasites are always few in numbers and for long periods in the disease lie inactive so that detection is usually a very difficult matter, and diagnosis by microscopical examination is possible only in rare cases.

Serum diagnosis.—The serum of dourine infected animals possesses certain specific properties which can be determined by (1) the complement-fixation test, (2) the agglutination test, and (3) the precipitin test. Of these, the first named is recognized as a delicate, safe and satisfactory method of diagnosis. The full technique and description is given further on (Appendix No. 2) together with some observations on the incubation period and longevity of dourine indicated by the serum reaction in the complement fixation test.

The agglutination and precipitin tests^{1, 2} are useful aids to diagnosis but less satisfactory than the complement fixation method especially when large numbers of animals are involved.

¹ Winkler & Wschelesky. *Berlin, Tierarztl. Woch.*, Dec. 31, 1911.

² Watson, *Proc. Amer. Vet. Med. Assoc.*, 1912.

APPENDIX No. 2.

DOURINE AND THE COMPLEMENT-FIXATION TEST.

The full description and detailed technique of the complement-fixation test method of diagnosis was published in 1915 in "Parasitology," and is reprinted in this appendix.

Among the animals experimentally and naturally infected with dourine are some which have survived the whole period of these investigations, 1905 to 1920, some which showed tolerance and relative immunity for many years, some which succumbed to the disease and some which died from other causes (charts I, II and III). The sera of many of these animals were used as controls in the 40,000 serum tests made during the period 1912 to 1919, the records of these re-actions furnishing some remarkable data, summarized below in table No. I.

All the animals there listed (table No. I) excepting the last two, Nos. 180 and 182, were infected five to seven years before the complement-fixation test was available, so that the reactions for that period cannot be given. However, Nos. 180 and 182 were tested at intervals of a few weeks from the first to the fourth and fifth years and constantly gave positive reactions. Further, all other horses which succumbed to dourine in from one to five years, not included in this table but which were serum-tested at frequent intervals, gave constant positive reactions up to the time of death.

Normal horses and horses in different stages of swamp-fever infection, used year after year as "negative controls," never gave a positive reaction.

It may therefore be concluded that in dourine a positive reaction to the complement-fixation test is given in all cases up to the time of death from the disease, and up to the fifth year after infection in cases that recover, and continued in some such cases up to the seventh, ninth, eleventh and thirteenth year.

In experimental dourine of the horse and donkey the incubation period is determined by the complement-fixation test as ten to eleven days (table No. II).

TABLE No. I.—Showing the serum reaction (to dourine) in the Complement-fixation test for a number of years after infection.

Horse No.	Complement-fixation Test.	
	Positive.	Negative.
2 (Chart No. I).....	9th and 10th year.....	9th year onwards.
10 (" " I).....	5th to 11th year.....	8th year onwards.
13 (" " I).....	5th and 6th year.....	8th year onwards.
17 (" " I).....	7th to 13th year.....	6th year onwards
24 (" " I).....		9th " "
29 (" " II).....		8th " "
71 (" " II).....	7th year.....	7th " "
26 (" " III).....		7th year onwards
48 (" " III).....		7th " "
52 (" " III).....		7th " "
66 (" " III).....		7th " "
67 (" " III).....		7th " "
69 (" " III).....		7th " "
70 (" " III).....		7th " "
71 (" " III).....		7th " "
75 (" " III).....		7th " "
82 (" " III).....	7th and 8th year.....	7th " "
180 (" " III).....	9th and 10th year.....	7th " "
182 (" " III).....	1st to 4th year.....	7th " "
	1st to 5th year.....	

TABLE No. II.—Showing the incubation period of dourine as determined by the Complement-fixation Test.

Horse No.			
190 (Table No. III)	1st positive reaction,	11th day after in	fection.
130 (" No. III)	" "	10th "	
Donkey			
183 (Table No. III)	" "	10th "	" "

DOURINE AND THE COMPLEMENT-FIXATION TEST.

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INTRODUCTION.

This paper is written with the purpose of drawing further attention to the value of the complement-fixation reaction as a diagnostic test in dourine and of recommending a method of procedure and technique arrived at with an experience of 15,000 tests for dourine made at the Veterinary Research Laboratory, Lethbridge.

In a previous paper I have briefly described the serum reactions in dourine. Since that paper was published in 1912 (*Proceedings of the Amer. Vet.-Med. Assoc.*) diagnostic tests for dourine have been carried along continuously at this laboratory, together with exhaustive control and experimental tests and the searching out of every possible source of error. By the numbers of horses available for experiment, the prolonged trial of the test through every known phase of the disease and its widest application in naturally occurring outbreaks, coupled with observations in company with the veterinary officers in charge of the field work, the complement-

fixation reaction has been thoroughly established as a sure, safe and specific method of diagnosing dourine. The experience shows that the test meets every requirement with regard to specificity, uniformity and decisiveness. It has been adopted as the official test for dourine in this country by Dr. F. Torrance, Veterinary Director General for Canada, who kindly permits me to publish this paper.

By the complement-fixation test it has been possible— and without difficulty— not only to make a certain diagnosis of the more or less symptomatic cases, but, and of greater importance, to positively determine the existence of the non-clinical, obscure and latent forms of infection.

Only by a systematic application of the test to every animal exposed to infection and in no other way known at present— can the healthy-looking, so-called immunocarriers of dourine be detected. When it is remembered that horses may tolerate dourine infection for periods of one to three years and remain for that time normal in general health and appearance but capable at times of transmitting the disease, the necessity of an early and definite diagnosis is evident. The complement-fixation test furnishes this and thus becomes of great importance as a basis for the control and suppression of dourine. It is being applied in every known outbreak of dourine in Canada, and, as a precautionary measure, in the various studs and to stallions standing for service in the districts that have come to be considered as dourine-infected areas.

Brief Explanation.

The general principles and mechanism of the complement-fixation reaction are now so widely known that it seems unnecessary for the purposes of this paper to repeat them in detail, a few remarks on the subject sufficing to make it clear and intelligible.

When an antigen is introduced into an animal either by way of natural infection or by artificial administration a group of reaction products arise in the animal serum—known as antibodies—bearing a specific relationship to the antigen and able to combine with it outside of the animal body under certain conditions. Micro-organisms, foreign blood cells and sera, albumens and many forms of protein matter are able to act as antigens. Thus an animal infected with dourine produces antibodies resulting from and specifically related to the dourine antigen, namely *Trypanosoma equiperdum*, the actual cause of the disease.

In a similar manner, an animal which has received injections of the blood of another animal species becomes possessed of antibodies having a specific affinity for the blood of that particular species of animal. In other words, the antibody arising in response to the exciting antigen in the process of infection, sensitization, or immunization, has the specific function of acting upon the antigen to neutralize it or prepare it for destruction.

The complement-fixation test applied in the diagnosis of disease consists of two sets of antigen and antibody, that is, two distinct and separable combining groups having no relationship to one another but in each of which Complement—a constituent of normal serum—is an essential factor. It is convenient to distinguish these groups by referring to the one comprising haemolytic serum, red cells and complement as the "haemolytic system" and to the other—closely related to the disease—comprising dourine, antibody corresponding antigen, and complement as the "antibody-combining group." Thus:—

	Haemolytic serum	} <i>Haemolytic system</i>
	Red cells	
	—COMPLEMENT—	}
Dourine or antibody combining group	Antigen	
	Antibody	

Before the test can actually be applied the exact dosage of the different elements in each group must be worked out by careful quantitative titrations—the most important step in the whole proceeding—and the operator must be absolutely assured that each group reaction is under his perfect control and that the least disturbing factor will be known to him. In the actual test only one complement unit is employed (the minimal amount necessary for the completion of the haemolytic system) so that only one of the reaction groups can come into operation; it is according to whether the complement unit is attracted and affixed to the antibody-combining group or to the haemolytic group that we obtain a positive or a negative reaction. The former will always be effected when the antigen and antibody correspond, that is, when the serum tested contains the specific reaction products of dourine infection even though in minutest quantity, so delicate is the reaction. Neither the antigen alone nor the serum alone, when properly prepared, can take up the complement unit; to do so, all three factors must be brought into intimate contact, and when the test serum does not contain specific dourine antibodies the complement is not fixed to this group, but remains free to join with and complete the two factors of the haemolytic system, so that the red cells undergo haemolysis and a negative reaction is indicated.

TECHNIQUE AND PROCEDURE RECOMMENDED.

Apparatus required.—Heavy glass tubes without lip, 5 inches by $\frac{1}{2}$ of an inch, and racks to hold twenty-four tubes in a double row, one above the other. Small test tubes, 4 inches by $\frac{1}{2}$ of an inch, for serum inactivation. Finely graduated measuring pipettes of 0.1, 1.0, 5.0 and 10.0 c.c. capacities. Graduated cylinders of 50 and 100 c.c. capacities. Erlenmeyer flasks of heavy glass, standard sizes up to 500 c.c. capacity. Large centrifuge cups and small centrifuge tubes. Ampoules and vials. A high power centrifuge machine, large water bath, and incubator room.

All glassware is sterilized by dry heat.

Diluting, washing and preserving fluids.

- (1) Normal salt solution—0.9 per cent pure sodium chloride in freshly distilled water. A large quantity should be made up (5000 c.c.) and sterilized in flasks having a siphon attachment.

- (2) Citrated salt solution:

Normal salt solution.....	100.0
Sodium citrate.....	1.5

- (3) Preserving fluid for trypanosomes:

Normal salt solution.....	90.0
Pure neutral glycerine.....	10.0
Formalin (Schering's).....	0.1

- (4) Preserving fluid for serum:

Glycerine.....	95.0
Phenol.....	5.0

I. PREPARATION OF REAGENTS.

A. *The Haemolytic System.*

(a) *Red Cells.*—A quiet sheep may be bled in the standing position, otherwise it should be placed upon its back in a V-shaped trough and held there by the attendant, an assistant shaving the neck and preparing the site of operation. The operator draws from the jugular vein, under aseptic conditions, 50 c.c. (more or less) of blood into a flask containing glass beads and in which the blood is defibrinated. It is then run through a double layer of fine, sterilized gauze into large centrifuge cups, about 20 c.c. of blood in each, adding three to four times the amount of salt solution. The corpuscles

are thrown down by centrifugal force, the upper fluid taken away and replaced with fresh salt solution, and the mixture again centrifuged. Washing in this way is repeated three times, when the red cells are carefully measured and suspended in an equal amount of salt solution, this 50 per cent stock suspension being stored in the ice chamber until needed.

(b) *Haemolytic Serum*.—Rabbits have a variable amount of natural haemolytic amboceptor for sheep's corpuscles—0.1 c.c. of fresh rabbit serum will usually haemolyze a like amount of 5 per cent corpuscle suspension. For test purposes a serum with a much higher haemolytic index is required and to obtain this rabbits are hypersensitized or immunized by repeated injection of sheep's corpuscles until a serum is given showing a haemolytic index of 0.0005.

Not less than six large healthy rabbits should be selected for the immunization, for one or several are apt to die from shock during the process. The rabbits are injected intraperitoneally with a first dose of 2.5 c.c. of the 50 per cent stock suspension of sheep's corpuscles. Every four to five days a further injection is given, each time increasing the dose until, after five or six injections, it has reached 10 c.c. This dose is repeated once or twice. After the sixth or seventh injection 5.0 c.c. of blood is drawn from the heart of each rabbit, using a hypodermic syringe and a fine needle. The operation can easily be performed and does no harm to the animal.

The serum of each rabbit is then heated for one-half hour at 56° C. and the haemolytic index established by titration (*vide* p. 22). It will be found, probably, that in only two or three rabbits out of six can the haemolytic index be raised to the desired degree, namely, 0.0005 or better. From such rabbits as much blood is drawn from the heart as will not endanger the life of the animal—about 25 c.c. The rabbits are then kept in reserve and can easily be reimmunized as required.

Finally, the serum is separated from the corpuscles and stored in very small ampoules—0.2 c.c. in each ampoule for convenience and economy—in the ice chamber.

When the serum is not to be used immediately it requires neither inactivation nor carbolization, and is, in fact, better without, the index remaining constant or but very slightly lowered even after six months. But unless the serum has been collected under aseptic conditions, rather than risk it spoiling, 1.0 c.c. of the carbolized glycerine preservative is added to 9.0 c.c. of serum before measuring it into the ampoules.

The whole procedure of immunizing rabbits, drawing blood from the heart, separating and bottling serum, can and should be carried out under aseptic conditions.

(c) *Complement*.—Normal guinea-pig serum in a fresh state furnishes a rich complement. Blood may be drawn from the heart, if desired, but as guinea-pigs are usually plentiful at a laboratory it is simpler to anaesthetize the animal in an ether jar, remove and suspend the guinea-pig over a centrifuge tube of 25 or 30 c.c. capacity, sever the arteries and veins on one side of the neck, and collect all the blood.

Centrifuge immediately, before coagulation takes place. The clear serum is taken off and placed in the ice chamber. Complement is always better used in the fresh state so the guinea-pig should not be bled until just before complement is needed for a titration or a diagnostic test.

B. *Dourine (antibodu) combining group.*

(x) *Antigen*.—A stock dourine antigen is obtained as the result of inoculating a number of white rats with *Trypanosoma equiperdum*, collecting the rat's blood when teeming with trypanosomes, and separating the trypanosomes from blood cells and serum by washing and centrifuging.

The blood of a dourine infected rat is collected in a vessel containing sufficient salt solution to prevent coagulation. Not less than ten large white rats—twenty or twenty-five rats, if a considerable amount of antigen is needed—are inoculated intraperitoneally with the diluted blood, injecting an equal amount, about 0.3 c.c., into each rat. This may be done very conveniently by taking a small sharp-pointed pipette, with rubber tubing and mouthpiece attached, drawing the blood up to a point marked by

a file or pencil, and expelling it into the abdomen, repeating the process with the same pipette for each rat. The object is to have all the rats come down together with a heavy infection. In the ordinary course a white rat dies of dourine between the end of the third and the beginning of the fifth day of infection. When twenty-five rats are inoculated at the same time about fifteen of them show a heavy trypanosome infection at the end of the third day, the remainder within the next 12-24 hours. It is necessary to make a rapid cover-glass examination of the blood of each rat forty-eight hours or so after inoculation and to sort the animals according as they show a light or a heavy infection into two or more groups. The result of the first blood examination will indicate approximately the time for a second examination and upon that the hour for bleeding may be judged. The timing of this operation is important for in the last six or eight hours of infection the trypanosomes multiply enormously, and if the rats are left until well on into this stage a very rich antigen will be furnished. Careful timing, however, is necessary, for it may easily happen that eight or ten rats will all die within one to two hours, if left too long. The bleeding should be carried out as rapidly as possible. The writer's method is simple and effective and may be worth describing in detail:

A running noose is made out of a two-foot length of thin copper wire, doubled over in the middle and twisted to the ends, the ends being passed through the ring formed at the beginning of the twist to form the noose and attached to any convenient fixture over a laboratory wash basin, six inches above an operating board resting across the basin. An ether jar, a flask of citrated salt solution, two sterile covered beakers and a razor complete the outfit.

An assistant passes the rats one at a time into the ether jar and hands them over as required. The animal is held back downwards in the left hand of the operator whose index or middle finger presses on the left front limb of the rat. The noose is slipped over the head and arranged so that the pull stretches the left side of the neck bending the head slightly to the opposite side, backwards and downwards. A beaker half filled with citrated salt solution is placed in position under the neck, the arteries and veins on that side and close to the shoulder then severed with a single sweep of the razor. Usually, the animals bleed better if one avoids severing the trachea. In this way ten rats may be bled in half-an-hour. The volume of blood and citrated salt solution should be about equal or a slight excess of the latter. The mixture is then passed through a double layer of sterile gauze to remove any small clots and fibrin into narrow centrifuge tubes, 10 mm. diameter and 10 c.c. capacity (when wider tubes are used it is more difficult to separate the trypanosomes and the wastage is greater). Centrifuge not longer than four to five minutes at 1,500 revolutions per minute so that the bulk of the corpuscles are thrown down while the trypanosomes remain in suspension. Draw off the cloudy suspension fluid into fresh tubes, then the upper layer of corpuscles—more or less mixed with trypanosomes—into another tube, and the next layer into a second tube, adding citrated salt solution and again centrifuging for 8-10 minutes. Draw off and discard as much of the upper fluid as appears clear and free from trypanosomes. Then collect from each tube into a single tube the upper pure white layer of trypanosomes, in another tube the middle layers slightly soiled with blood, and in a third and fourth tube the lower layers in contact with the blood cells. Add normal salt solution now, not citrate, shake up well and centrifuge again, repeating the washings until all the trypanosomes are obtained in a pure white mass.

Ten rats bled at the right time will furnish 5.0 c.c. of trypanosomes. Twice the volume of the glycerine-formalin preservative is added and the mixture stored in sealed amber ampoules, 1.0 c.c. in each, in a block of ice.

5.0 c.c. of trypanosomes will make 100 c.c. of antigen, sufficient for more than 500 diagnostic tests. The antigen will keep indefinitely if solidified by freezing, and for 6-8 weeks or longer when stored in liquid form, in sealed ampoules, on ice.

(y) *Antibody*.—In the diagnostic tests the antibody, of course, is or is not present in the suspected serum. But for purposes of control and titration and to thoroughly understand the combining action of dourine antigen and antibody it is

absolutely necessary to have one or more series of known positive or specific dourine horse sera, of which the antibody content can be determined. To obtain this a horse is inoculated with *Trypanosoma equiperdum*. Ten days later and at weekly intervals thereafter, blood is drawn aseptically from the jugular vein, the serum collected and tested for antibody content (*vide* p. 28). A series of specific positive sera are thus obtained, representative of different periods and stages of the disease. Stored in the ice chamber the sera will retain their specific properties for many months, even years, if collected sterile. If not absolutely sterile the serum may be preserved by adding 1.0 c.c. of 5 per cent carbolyzed glycerine, or the same amount of iodized glycerine to 9.0 c.c. serum. At the same time one should collect and store a number of negative control sera under the same conditions.

II. TITRATION OF REAGENTS.

(1) Titration of Haemolytic Serum (*Amboceptor*).

Prepare the following stock dilutions of serum and corpuscle suspension:—

1. Haemolytic serum (rabbit anti-sheep).....	0.1 cc.
Normal salt solution (0.85 per cent).....	9.9 "
	10.0 " 1:100
2. Complement:	
Fresh guinea-pig's serum.....	1.0 "
Normal salt solution.....	19.0 "
	20.0 " 1:20
3. Corpuscle suspension:	
Washed sheep's corpuscles (50 per cent stock suspension).....	2.0 "
Salt solution.....	23.0 "
	25.0 " 1:25

Further dilutions of the haemolytic serum are made as under:—

Tube No.	Salt solution c.c.	Haemolytic serum c.c.	
1	3.0	1.0 (1:100) equals	1:400 (0.0025 serum in 1.0 c.c.)
" 2	5.0	1.0 "	1:600 (0.0016 " ")
" 3	7.0	1.0 "	1:800 (0.0012 " ")
" 4	9.0	1.0 "	1:1000 (0.001 " ")
" 5	0.5	1.0 (1:1000) "	1:1500 (0.00066 " ")
" 6	1.0	1.0 "	1:2000 (0.0005 " ")
" 7	2.0	1.0 "	1:3000 (0.00033 " ")
" 8	3.0	1.0 "	1:4000 (0.00025 " ")
" 9	4.0	1.0 "	1:5000 (0.0002 " ")

In each tube 1.0 c.c. only of the dilution is held back, the excess amount being discarded, 1.0 c.c. each complement and red cell suspension added, which with 2.0 c.c. salt solution make a total volume of 5.0 c.c. in each tube.

The complete titration set is then:—

	Serum dilutions (as above)	Salt solution	Complement 1:20	Red Cell suspension	
	c.c.	c.c.	c.c.	c.c.	
Tubes 1-9	1.0	2.0	1.0	1.0	Titration set
Tube No. 10.....	1.0	3.0	—	1.0	Serum control (1:100 dil.)
" No. 11.....	—	3.0	1.0	1.0	Complement control
" No. 12.....	—	4.0	—	1.0	Red cell control

Mix well and incubate for two hours at 37° C.

The control set, tubes 10, 11, and 12, must not show any trace of haemolysis.

The *titre* of the haemolytic serum is indicated by the amount present in the *last* tube of the series 1-9 in which dissolution of all the red cells is *complete*, that is, the least amount necessary to dissolve a definite amount of red cells.

For example, if in tubes Nos. 1-6 haemolysis is complete, not quite complete in tube No. 7, and still less in Nos. 8 and 9, then the titre is the amount of serum in tube No. 6, or 1.0 c.c. of a 1:2000 dilution, 1 unit being expressed at 0.0005.

A serum with a unit value of between 0.0002-0.0005 is quite satisfactory, but when the value of a single unit exceeds the latter amount the results are not so good.

The relationship and combined action of haemolytic amboceptor and complement should be clearly understood. To do this a number of experimental tests should be undertaken, using in one series only one unit of amboceptor with fractional amounts of complement, in another series two units of amboceptor and lesser complement fractions, four units and so forth, progressively multiplying the number of amboceptor units while further reducing the fractions of complement. It will be found, for instance, that two units of amboceptor require a lesser amount of complement than one unit to completely haemolyse a standard amount of red cells. The lesser the amount of complement that can be safely employed in the practical tests the more delicate becomes the fixation reaction, the equilibrium of the haemolytic system being more easily upset, even by a test serum naturally weak in antibody content and which, if a relatively large complement unit was employed, might be insufficient to give a complete reaction. On the other hand the reduction of complement must not be carried to such an extreme point that any slightly inhibitive property of one of the other reagents would tend to obscure it and give a false fixation.

It is essential that for all subsequent titrations and tests a standard dose of haemolytic amboceptor be fixed and rigidly adhered to. For all practical purposes the use of two units of amboceptor permit of a sufficiently fine gradation of complement, while still allowing a margin of safety. The dose is therefore fixed constantly at two units, to which complement is always titrated as in the next procedure.

(2) Titration of Complement.

Prepare (1) a stock dilution of guinea-pig complement and (2) a suspension of sheep's corpuscles, as in the previous titration.

Also (3), an haemolytic serum (amboceptor) dilution, so that 1.0 c.c. of the diluted serum contains two amboceptor units. For example, if the value of one unit is 0.0005, then 0.001 will be that of two units, the dilution being accordingly 1:1000.

The titration of complement is of the utmost importance and requires the greatest accuracy, as already indicated. Until one has become familiar with the technique and expert in reading the reactions the titration is best carried out in a double set, the second having one-half of the amount of each reagent used in the first set, the one serving as a check to the other.

The two sets are arranged as follows:—

Titration of Complement.

Tube No.	First Set			Second Set		
	Salt solution	Complement	Haemol. serum	Salt solution	Complement	Haemol. serum
1	2.0	0.3 (1:20)	1.0	1.0	0.15 (1:20)	0.5
2	2.0	0.4	1.0	1.0	0.2	0.5
3	2.0	0.45	1.0	1.0	0.225	0.5
4	2.0	0.5	1.0	1.0	0.25	0.5
5	2.0	0.55	1.0	1.0	0.275	0.5
6	2.0	0.6	1.0	1.0	0.3	0.5
7	2.0	0.65	1.0	1.0	0.325	0.5
8	2.0	0.7	1.0	1.0	0.35	0.5
9	2.0	0.8	1.0	1.0	0.4	0.5
10	2.0	1.0	0.5	1.0	0.5	0.25
11	3.0	—	1.0	—	—	—
12	3.0	1.0	—	2.0	—	—

Add 1 c.c. red cell suspension to each tube.

Add 0.5 c.c. red cell suspension to each tube.

Mix well (avoiding undue frothing).

Incubate at 38-39° C.

Agitate the mixtures again by shaking the racks after ten minutes incubation.

Read the reactions one hour later.

Tube No. 10 controls the original haemolytic titration, only one unit of amboceptor being used with an excess of complement. In this tube complete haemolysis should occur.

Tube No. 1 will show only slight or partial haemolysis; as one descends the series the reaction is seen to be increased, until, usually between Nos. 4 and 7, a tube is reached in which the reaction is absolutely complete. The first tube in the series in which *all* the red cells are completely dissolved indicates the complement titre. If this occurs in tube No. 5, for example, then 0.55 c.c. of a 1:20 dilution of complement is the titre, equivalent to 1.0 c.c. of a 2.75 per cent dilution.

For the antigen titration and final tests the complement is accordingly made up so that 1.0 c.c. of the dilution contains the amount of complement indicated by the above titration.

From now on it is optional whether one employs the relatively large amounts of reagents as given in the first set of complement titration, or the one-half amounts as in the second set. The latter is the more economical, especially when a large number of tests are being performed, and is given personal preference to by the writer as it seems to provide an even more highly sensitive test reaction than when the larger amounts are employed.

(3) Titration of Antigen.

Dilute 1.0 c.c. of stock trypanosome antigen with 19.0 c.c. of normal salt solution.

Prepare the complement and haemolytic serum according to their titration values already determined.

Inactivate by heating for half-an-hour at 58° C. in a water bath, 2.0 c.c. of known positive dourine horse serum and 2.0 c.c. of known negative or normal horse serum.

The antigen is then titrated in a double set, the one being with the positive serum, the other with double the amount of negative serum. Thus:—

Positive set					Negative set			
	Salt sol.	Known positive horse serum	Antigen	Complement	Salt sol.	Known negative horse serum	Antigen	Complement
Control	c.c.	c.c.	c.c.	c.c.	c.c.	c.c.	c.c.	c.c.
Tube No. 1	1.0	0.1	0.02	0.5	1.0	0.2	0.05	0.5
" 2	1.0	0.1	0.05	0.5	1.0	0.2	0.1	0.5
" 3	1.0	0.1	0.01	0.5	1.0	0.2	0.2	0.5
" 4	1.0	0.1	0.15	0.5	1.0	0.2	0.3	0.5
" 5	1.0	0.1	0.2	0.5	1.0	0.2	0.4	0.5
" 6	1.0	0.1	0.25	0.5	1.0	0.2	0.5	0.5
" 7	1.0	0.1	0.3	0.5	1.0	0.2	0.6	0.5
" 8	1.0	0.2	—	0.5	1.0	0.2	—	0.5
" 9	1.0	—	0.25	0.5	1.0	—	0.5	0.5
" 10	1.5	—	—	0.5	2.0	—	—	—

Mix well and incubate for one hour and ten minutes at 38-39° C.

Mix together equal quantities of haemolytic serum (amboceptor) and red cell suspension, then add 1.0 c.c. of the mixture to each tube.

Shake again and incubate for two hours longer.

It is usually possible to read the antigen titre in 1½ hours and proceed with the final tests; nevertheless, the tubes should be left or replaced in the incubator for the full two hours and then put on one side for further reference and to see if any further action has taken place.

Tube No. 10 is the control for the haemolytic system and must show complete haemolysis. No. 10 in the second set contains only haemolytic amboceptor and red cells and must not show the slightest degree of haemolysis. No. 8 controls the horse serum, No. 9 the antigen, the red cells being haemolysed in all.

The positive set will show more or less complete fixation of complement—no haemolysis, except perhaps in the first and second tubes, the negative set complete haemolysis. When the antigen appears very strong there may be some inhibition in the negative set in the tubes containing the larger amounts of antigen.

The amount of antigen to be selected as the titre for the final tests is that which gives complete fixation with the positive serum while double the quantity in the corresponding tube of the negative set does not prevent or inhibit haemolysis.

III. THE SERUM TO BE TESTED.

Collection of serum.—The chief point aimed at in collecting blood from suspected animals is sterility, especially when the specimens have to be transported over long distances and mailed to the laboratory. Absolute sterility is not essential, nevertheless as near as possible aseptic conditions are to be strongly recommended and the avoidance of adding carbolic acid or any other antiseptic fluid to the sample specimen as a preservative. The blood clot should be well formed and the serum odourless and clear or only slightly tinged with haemoglobin.

The condition of a sample of blood may vary greatly according to the size and shape of the vial or tube containing it, the slowness or rapidity with which blood is run into the vial, the partial or complete filling of the vial, the shaking of the specimen before coagulation has occurred, and in other ways irrespective of aseptic conditions and of abnormal properties of the blood itself. In square or rectangular bottles and in specimen vials without a neck the clot has a tendency to cling firmly to the sides, the serum being separated with more or less difficulty. In small round bottles, curved into a narrow neck and mouth, for corks, filled with freely flowing blood to within a margin of the narrowest diameter but not touching the cork, and allowed to stand for at least half an hour for coagulation, there is usually an abundance of clear serum.

Such bottles, of one ounce capacity, one inch in diameter, three-eighths inch neck and mouth, are very suitable for field work. They must be absolutely clean and free from any trace of soap, alkali or acid. These bottles are distributed from this laboratory after being sterilized in the hot air oven, corked, labelled and well wrapped in sterile paper wrappers. Also, large bore needles attached to three inches of rubber tubing with a small glass nozzle, separately wrapped and sterilized. With this simple apparatus and observing the usual precautions during operation it is an easy matter to draw blood from the jugular vein of a horse, aseptically.

Among the last 6,000 samples of blood secured in this manner less than twenty have reached the laboratory in a condition unfit for testing and these few unfit specimens have been ten days or more in transit.

On reaching the laboratory the specimens are briefly examined and where necessary the clots are detached from the sides of the bottles with a sterile wire. They are then left to stand in a cool chamber overnight for the serum to clear. The serum is then drawn off into small test tubes, about 2.0 c.c. in each, and is ready for inactivation.

Inactivation of serum.—Before any specimen of horse serum can be used in the complement fixation test it has first to be inactivated. All animal serum in a very fresh state contains complement in a varying amount. This constituent is readily destroyed by heating the serum to 55-56° C. for one half hour. No complement other than that employed in the haemolytic system may take part in the reaction. As a matter of fact horse complement very rapidly becomes inert and in specimens several days old is a practically negligible quantity. However, in normal horse serum there arise several other factors which, unless destroyed or

rendered inactive, are able to act upon complement and antigen and disturb an haemolytic system. All untreated horse, donkey and mule sera possess enzymotic and proteolytic properties, potentially at least, and becoming active in sera a day or two old. They act upon most preparations of antigen, especially upon macerated organs, such as the liver and spleen, and are all more or less anticomplementary, more so in the presence of antigen than without it. Such action, of course, is non-specific and must be eliminated, otherwise it would be difficult or impossible to distinguish a specific from a non-specific reaction. Fortunately it can be eliminated, and the equilibrium of the serum fixed, by a proper and sufficient inactivation. *It is more resistant to heat than is complement and is not wholly destroyed at 50° C.* This is an important point, and one that appears to have been overlooked. I cannot help thinking that it is the explanation and the source of error of many of the apparent failures or discrepancies, especially that of non-specific fixation, which some serologists experience. A reference to the literature on complement-fixation methods shows a remarkable lack of uniformity in respect to the degree of heat and the length of time for the inactivation of suspected sera—fifteen to thirty minutes at degrees varying between 50 and 58° C.

A few experiments with sets of ten or twenty different horse, mule and donkey sera, each set being heated for thirty minutes at different degrees between 50 and 62° C. and then tested in the haemolytic system, with and without antigen, will show the importance and necessity of a very careful inactivation and the temperature required (*vide p. 27*).

Method of inactivation recommended.—A water bath, sufficiently large to hold 200 small test tubes, is heated to 60° C. The tubes, containing 2.0 c.c. serum in each (numbered for identification in waterproof india ink, labels being apt to become detached), are placed within the inner tank which is to contain sufficient water to mount to the level of the serum or to about half the height of the tubes. The cover of the tank should have two perforations for thermometer tubes which are inserted into control tubes within the tank, enabling the temperature to be read without removing the cover. Another thermometer passes directly into the outer tank. For the first few minutes the temperature will rapidly fall; it is brought up to 59° again—taking about ten minutes—and maintained at that point for a full half hour, for horse serum, and to 62° for one half hour for donkey or mule serum.

There is no danger of destroying the specific antibodies of dourine sera by heating to the points given. Dourine sera can, in fact, be heated up to 65°, or to the point of coagulation, and still retain an active antibody content to give the test reaction, but the anticomplementary and non-specific factors in horse sera are wholly destroyed at 59°, and in donkey and mule sera at 62°.

To control the inactivation, with each batch of suspected sera several known positive dourine sera as well as known (anticomplementary and non-specific) negative sera are included and all tested together in the final diagnostic test.

Experiment showing the degree of inactivation of suspected serum necessary for specific reactions.

Dose of serum, 0.2 c.c., unheated and heated at different degrees of temperature and tested with trypanosome antigen as in the diagnostic test.

Normal healthy horses	Unheated serum	Serum heated for one half hour at degrees Centigrade						
		50	54	56	58	60	62	64-65
No. 1	++++	++++	++	+	-	-	-	-
" 2	++++	++++	++	+	-	-	-	-
" 3	++++	++++	++	+	-	-	-	-
" 4	++++	++++	+++	++	-	-	-	-
" 5	++++	++++	++	(+)	-	-	-	-
" 6	++++	++++	++	(+)	-	-	-	-
" 7	++++	++++	+++	++	-	-	-	-
" 8	++++	++++	++	(+)	-	-	-	-
" 9	++++	++++	+++	(+)	-	-	-	-
" 10	++++	++++	+++	+	-	-	-	-

Inhibition. Non-specific.

Dourine horses					
No.	1st year of disease)				
" 2	(" ")	++++	++++	++++	++++
" 3	(" ")	++++	++++	++++	++++
" 4	(" ")	++++	++++	++++	++++
" 5	(" ")	++++	++++	++++	++++
" 7	(" ")	++++	++++	++++	++++
" 7 (2nd	" ")	++++	++++	++++	++++
" 8 (3rd	" ")	++++	++++	++++	++++
" 9 (4th	" ")	++++	++++	++++	++++
" 10 (5th	" ")	++++	++++	++++	++++

Specific complement-fixation.

Normal	Unheated	50	54	56	58	60	62	64-65
Mule No. 1	++++	++++	++++	+++	+	-	-	-
" 2	++++	++++	++++	+++	+	+	-	-
Donkey No. 1	++++	++++	++++	+++	+	+	-	-
" 2	++++	++++	++++	+++	+	-	-	-

Inhibition. Non-specific.

++++ See page 28 for the meaning of these reaction expressions.

Note.—In the non-specific inhibition reactions the red cells are loosely sedimented. In the specific complement fixation reactions the red cells are precipitated in a mass or agglutinated in lumps. When the sera are tested *without antigen*, as in the serum controls, the dourine sera, of course, give no specific reactions, but the inhibition reactions are given by normal and dourine sera alike when insufficiently inactivated, though to a lesser degree than when antigen is present.

Conclusion.—Suspected horse serum must be heated to at least 58° C. (59-60° for safety) and mule or donkey serum to 62° C., to eliminate non-specific reactions.

The Antibody content of Dourine Sera, and the dose of suspected Serum necessary for a Diagnostic Test.

The maximum dose of horse serum used in a diagnostic fixation test is 0.2 c.c. This amount is not exceeded for fear of any disturbance to the haemolytic system by the non-specific reactions which larger doses are apt to cause. Double the amount

can actually be used with perfect safety provided the serum is correctly inactivated. But it is unnecessary to use more than 0.2 c.c., for that amount of serum of a dourine horse will contain in the case of a serum very weak in antibody content at least one unit, and in the case of a serum strong in antibody ten, twenty, forty or more units—and one unit of antibody is sufficient to give a positive reaction with the fixation test.

That this is so may be determined by taking a series of sera collected from animals in active and in latent phases of the disease and titrating out each serum for antibody content.

The experiment is carried out as follows:—

The sera are first inactivated by heating for one half hour at 59° C. Three stock tubes are then taken for each serum, (1) containing the pure serum, (2) a dilution of 1:10, and (3) a dilution of 1:100, these dilutions permitting of the accurate measurement of the smaller doses.

Twelve tubes are now arranged for each serum to be tested—the first and last to contain 0.2 c.c. of undiluted serum, the largest amount used in the test, the last tube being the serum control without antigen, the intervening tubes to contain gradually decreasing doses of serum. Enough salt solution is then added to make up to 1.0 c.c. in each tube, then the antigen and complement in amounts previously determined by careful titration, and finally, after incubation for seventy minutes, haemolytic serum and red cells—as in a diagnostic test.

An experiment of this kind is given below, the titres of seven sera from different horses in different phases of the disease being determined.

Experiment for determining the Antibody content of Dourine Sera by the Complement Fixation Method.

Dose of inactivated dourine serum	Complement fixation reactions with dourine serum.						
	No. 1	No. 2	No. 3	No. 4	No. 5	No. 6	No. 7
c.c.							
0.2 (undiluted, standard doses)	++++	++++	++++	++++	++++	++++	++++
0.15	++++	++++	++++	++++	++++	++++	++++
0.1	++++	++++	++++	++++	++++	++++	++++
0.075 (0.75 of 1:10)	++++	++++	++++	++++	++++	++++	++++
0.05 (0.5 " ")	++++	++++	++++	++++	++++	++++	++++
0.025 (0.25 " ")	++++	++++	++++	++++	++++	++++	++++
0.01 (0.1 " ")	++++	++++	++++	++++	++++	++++	++++
0.0075 (0.75 " 1:100)	++++	++++	++++	++++	++++	++++	++++
0.005 (0.5 " ")	++++	++++	++++	++++	++++	++++	++++
0.0025 (0.25 " ")	++++	++++	++++	++++	++++	++++	++++
0.001 (0.1 " ")	++++	++++	++++	++++	++++	++++	++++
0.2 (serum control without antigen)	—	—	—	—	—	—	—
Indicated value of one antibody unit	0.005	0.005	0.0075	0.01	0.05	0.025	0.2
Number of antibody units in 0.2 c.c. of dourine serum—maximum dose	40	40	26½	20	4	8	1

++++ means complete fixation of complement—absolutely no trace of haemolysis. Red cells more or less clumped. A very strong positive reaction.

+++ is also a strong positive reaction, with just a faint trace or tinge of haemolysis.

++ is a rather weak positive, indicating partial fixation—about one-half the red cells haemolysed.

+, a very weak or faint positive—slight fixation, with more than one-half the red cells haemolysed.

—, a negative reaction. Complete haemolysis of red cells.

The smallest dose of serum which combines with antigen to cause complete fixation (++++) indicates the value of one antibody unit, and from this may be calculated the number of units in the standard or maximum dose and the value of a dourine serum in antibody content.

Such values are more relative than absolute, for the titre of a dourine serum may be somewhat higher or lower according to the amount of dourine antigen present and the fineness with which the haemolytic system has been adjusted—just as the titre of the haemolytic serum itself is correlative to the amount of complement and red cells (antigen).

The seven sera, Nos. 1-7, used in this experiment are taken from dourine control horses in the first, second, third, fourth, fifth, sixth and seventh year of the disease, respectively. No. 1 from a mare showing clinical symptoms; No. 2 from a stallion showing occasional symptoms; Nos. 3 and 4 from mares very rarely showing symptoms and progressing towards recovery; Nos. 5, 6 and 7 from mares that have not shown any symptoms for three, four and five years respectively, and which have made complete recovery, been bred to a healthy stallion each year—without transmitting infection—and raising healthy offspring.

In addition to the above, among our experimental horses that have recovered from dourine, there are two mares that give a positive (+++++) reaction with 0.2 c.c. serum after six years, and one mare a positive (++++) after seven years. On the other hand, there are three mares that have entirely ceased to react, even with two or three times the amount of serum, after six to seven years of recovery, although they reacted positively up to the fifth year.

Conclusion.—0.2 c.c. of horse serum from a dourine infected animal contains up to forty units of specific antibody. In the case of horses that have completely recovered from dourine and which are no longer able to transmit the disease, one or several units of antibody are present in the same amount of serum up to the fifth year of recovery. After that period they may cease to react—indicating that not only was an absolute recovery made but that the immunity was lost in about five years (proof of which has been given by inoculation experiments with *T. equiperdum* on recovered horses).

For diagnostic tests it is sufficient to use three doses of serum, namely, 0.2, 0.15 and 0.1 c.c.

The first appearance of a positive serum reaction in dourine infections.

Having fixed upon a standard dosage of suspected serum, it is now necessary to know the incubation period of dourine and when a first positive serum reaction may be expected, for otherwise a negative reaction would be valueless or even misleading.

In this connection there follow the records of some experiments:—

Experiment for determining the length of time between dourine infection and the first appearance of a positive serum reaction.

A healthy filly 2½ years old, was infected with dourine by smearing over the vaginal mucosa a few drops of blood containing *Trypanosoma equiperdum*.

Serum was collected from this young mare before infection and daily up to the fifteenth day after infection, and tested by the complement-fixation method, with trypanosome antigen. The results were as follows:—

Dose of serum	Before infection	Days after infection					
		1 to 10	11	12	13	14	15
c.c.							
0.2	—	—	+++++	+++++	+++++	+++++	+++++
0.15	—	—	+++	+++++	+++++	+++++	+++++
0.1	—	—	+	+++++	+++++	+++++	+++++
0.05	—	—	—	++	++	++	++
0.01	—	—	—	—	—	+	++
0.005	—	—	—	—	—	—	+

Thus, the first appearance of a positive serum reaction was eleven days after infection.

In three earlier experiments of this kind, but in which serum was not collected for testing until the twentieth day after infection, the reaction in each case was strongly positive.

The incubation period of dourine in the light of the complement-fixation test is indicated, by the above experiments, as not less than eleven days and not over twenty days. However, the strain of dourine used in these experiments was of high virulence; when horses become infected with strains of low virulence—and there is much variation in dourine strains—the incubation period is probably prolonged.

A negative reaction should not be taken as final or conclusive when the interval between exposure to infection and the collection of test serum is less than two months.

IV. THE DIAGNOSTIC TEST.

Two methods of procedure are here recommended:—

- (1) When only one or several tests are to be made.
- (2) For daily routine testing or when 50, 100, or more tests are to be made at one time.

In either case a necessary preliminary is the titration of complement (*vide*, p. 166). This established, sufficient complement dilution is made up—0.5 c.c. of the dilution to contain the smallest amount indicated by titration—to do for the titration of antigen and for as many serum tests and controls as are to be made. It is advisable to make up an excess of complement rather than have a deficit, so as to use one stock uniform dilution throughout and avoid having to make up fresh stocks during the testing.

The trypanosome antigen is then titrated against a known positive dourine serum and a known negative serum (*vide*, p. 167).

First method of procedure—for one or several tests.

Four tubes and one pipette of 1.0 c.c. capacity, graduated 1–100, are needed for each serum to be tested. 1.0 c.c. salt solution is measured into each tube. In each set of four tubes 0.2, 0.15, 0.1 and 0.2 c.c. of the inactivated test serum is added. Antigen in the amount already decided by titration is now added to the first three tubes in each set, omitting it from the fourth tube which serves as a serum control. Complement, 0.5 c.c. of the dilution required, is then added to all tubes. Sets of positive and negative sera are included with the above, and, in addition, controls for the various reagents. For the reagent controls five tubes are needed: (1) Antigen control, omitting the test serum, (2) haemolytic control, omitting serum and antigen, (3) haemolytic serum control, omitting test serum, antigen and complement, (4) complement control, omitting test serum, haemolytic serum and antigen, (5) red cells control, containing only red cells and salt solution. The controls are made up to a uniform volume of 2.5 c.c. by adding salt solution as required.

When the test serum, antigen and complement have been mixed together, the tubes are incubated at 38–39° C. for 70 minutes.

Equal quantities of the haemolytic serum dilution and the red cell suspension (4 per cent) are mixed together and 1.0 c.c. of the mixture added to every tube excepting the last two controls, Nos. 4 and 5, to which 0.5 c.c. red cells only are added.

The tubes are again shaken and incubated for another two hours when the reactions may be read, a second reading being made the following morning, about twelve hours later, the racks being left at a cool room temperature meanwhile.

The above procedure is indicated in the following table:—

Table showing method of procedure for a diagnostic fixation test.

	Tube No.	Salt solution	Test serum	Antigen	Complement	Mixture of haemolytic serum and red cells	Fixation	Reaction
<i>Diagnostic set for each suspected serum</i>								
	1	c.c.	c.c.	c.c.	c.c.	c.c.	+	
	2	1.0	0.2	0.2	0.5	1.0	+	Strong positive
	3	1.0	0.15	0.2	0.5	1.0	+	Weak positive
	4	1.0	0.1	0.2	0.5	1.0	+	Weak positive
	5	1.0	0.2	—	0.5	1.0	—	Haemolysis
<i>Serum control</i>								
	1	1.0	0.2	0.2	0.5	1.0	+	Complete fixation
	2	1.6	0.1	0.2	0.5	1.0	+	Complete haemolysis
	3	1.0	0.2	—	0.5	1.0	—	Complete haemolysis
<i>Test control with known positive serum</i>								
	1	1.0	0.2	0.2	0.5	1.0	+	Complete haemolysis
	2	1.0	0.2	—	0.5	1.0	—	Complete haemolysis
<i>Reagent controls</i>								
(a) Antigen	1	1.0	—	0.2	0.5	1.0	—	Complete haemolysis
(b) Haemolytic system	2	1.0	—	—	0.5	1.0	—	No haemolysis
(c) Haemolytic serum	3	1.5	—	—	—	1.0	—	No haemolysis
(d) Complement	4	1.5	—	—	0.5	Red cells only	—	No haemolysis
(e) Red cells	5	2.0	—	—	—	0.5	—	No haemolysis

70 minutes at 38-39° C.

2 hours at 38-39° C. First reading of reactions.

12 hours at cool room temperature. Second reading of reactions.

Second method of procedure—for daily routine testing or when 50, 100, or more tests are to be made.

This is only a slight modification of the first method of procedure to allow of more rapid and less laborious work in testing large numbers of suspected sera.

Two test series are made, the first series, in which only one tube for each serum is used (instead of four tubes as before), containing the maximum dose, 0.2 c.c., and antigen, eliminating all negative sera and at the same time indicating the positive sera. These latter are again tested on the following day, using the four tubes—the three standard doses of serum and serum control—as in procedure No. 1, including them with the next lot of sera to undergo the first test in which the single tube is used.

If a negative serum does not give a fixation reaction with 0.2 c.c. serum it certainly will not with the lesser doses, and as a serum control is only needed in the case of a serum which fixes complement, the single tube is obviously all that is required to determine a negative serum. Further, the sera with which complement-fixation takes place in the one series serve as additional controls when included with and fully tested out in the second series—one day's work thus checking the other, continuously.

In routine testing at this laboratory, when large numbers of sera are being dealt with, it is the practice to make a repeat test with each serum negative at the first test and to arrange the work and the different series so that each day's tests include: (a) a series not before tested (one tube for each serum); (b) the sera tested the day before with negative result (one tube for each serum); (c) the sera tested the day before with fixation reactions (four tubes for each serum); and, in addition, the usual series of known positive dourine sera, negative controls and reagent controls.

All suspected sera are thus tested twice over so that if any error or omission in the technique has been made it will surely be indicated.

Interpretation of the reaction.—Fixation of the complement, not in itself visible in the test tube, is indicated by the prevention of haemolysis of the red cells and constitutes a positive reaction, on which a diagnosis of dourine is given.

When no complement is fixed the red cells are completely haemolysed and the reaction is then said to be negative.

The prevention or inhibition of haemolysis may be complete, partial or slight—according to the richness of the serum in specific antibodies. However, with the standard doses of serum, in the great majority of cases, the reaction is either clearly positive or clearly negative. Occasionally, complement fixation complete with 0.2 c.c. serum, partial with 0.15 c.c. and slight with 0.1 c.c. may be given. This is a positive reaction and indicates that the serum is weak in antibodies, only one unit being present in 0.2 c.c. serum.

Partial fixation with 0.2 serum and complete haemolysis with 0.1 serum is a rare reaction and of a questionable nature. In the serum controls, without antigen, haemolysis should always be complete. Very rarely indeed it happens that haemolysis in the serum controls is not complete, the mixture having a cloudy or opaque appearance and some of the red cells remaining unhaemolysed. This may be the result of insufficient inactivation or of changes in the serum due to certain bacterial growths. When such questionable reactions are given a fresh specimen of serum is asked for and a retest made.

GENERAL REMARKS.

The successful practice of the complement fixation test depends mainly upon the preparation and use of powerful reagents, their specificity and the accurate determination of their relative values, the fixing of standard doses wherever possible, and a constant, uniform technique and method of procedure.

Close familiarity with the activity of the reagents is essential for the best results.

Stock reagents should be prepared in quantities calculated to meet all requirements for as long a time as the activity of the reagents remains practically constant. Thus: sufficient haemolytic serum for six months' work; antigen to suffice for one month's work; fresh red cell suspension once a week; fresh complement daily or on alternate days, or as needed. It is advisable to use the blood of two sheep for sensitizing rabbits and to use the red cells of the same sheep for the haemolytic system.

The following points of extreme importance will bear repetition:—

(1) The amount of red cells in suspension must be very accurately measured and the standard amount never varied.

(2) The use of the least possible amount of complement which with two units of haemolytic serum causes complete haemolysis of red cells.

(3) The use of twice the amount of antigen which with a dourine antibody unit is necessary to fix the complement, provided the same amount of antigen alone has no inhibitory action.

(4) Careful control of the inactivation of suspected sera by known positive and known negative sera.

(5) Control of the diagnostic tests by a series of known positive sera, each having an antibody unit of different value, high to low.

DISCUSSION.

The reliability of the complement fixation test as a certain and specific means of diagnosis has been questioned, not, I think, very seriously or on strictly scientific grounds, but more in respect to its practical application and on an unwarranted supposition that it is still very imperfectly understood, that the technique and method of procedure is so intricate and laborious, that the reactions themselves are subject to and have to be guarded against so many possible disturbing influences that the adoption of such a method of diagnosis is attended with considerable risk.

Can the test be practically applied? Yes, without doubt, and with as much ease as a mallein or tuberculin test is applied. In the one case blood is collected in the field and sent in for a laboratory test; in the other the reagents are prepared in the laboratory and sent out for a field test. Further, as many retests can be made by the complement fixation method as desired, for no toxins or immunizing substances are injected into the suspected animal to interfere with subsequent diagnostic tests. This test is no longer a new departure in veterinary diagnosis; it is successfully applied in glanders, contagious abortion and in other specific diseases and is yearly coming into more general use.

Are the test reactions and the different factors concerned in them imperfectly understood?—Such a view is not held by serologists and can only be retained by those who have not the opportunity of closely studying the subject and becoming familiar with the finer points of it. Any attempt to apply the test by one who has not thoroughly mastered the technique and gained complete control of the reagents would, of course, be dangerous. But the complement fixation reaction furnishes the most perfect, biological, diagnostic test yet devised, one in which all adverse or disturbing factors can be eliminated and in which a clear knowledge of the properties and mode of action of the reagents has been ascertained,—far more so, in fact, than that of a mallein or tuberculin reaction which, in application and interpretation, is crude in comparison. The very delicacy of the fixation reaction and the strict laws and conditions governing it, add to the exactness, value and reliability of the test.

Is the technique too intricate and laborious?—Not more so than many other necessary and accepted laboratory methods, and this is essentially a laboratory test.

Is it necessary to use a pure suspension of trypanosomes as antigen?—By the employment of a pure suspension of dourine trypanosomes as antigen non-specific and false or misleading reactions are avoided. Many other ways of preparing antigen for the dourine test have been tried by different investigators but, with one exception, with little success. Mohler and Eichhorn recommend a spleen preparation of a rat dead from surra. I have used the spleens of rats dead from dourine in several thousand tests and with very good results, but, on the whole, such preparations are inferior to the trypanosome suspension and possess a number of disadvantages. Spleen preparations are often troublesome on account of a more or less anticomplementary action or owing to a weakness in specific antigenic property. They are very unstable and of inconstant value and give rise to many borderline or questionable reactions which can be eliminated or definitely decided by the trypanosome antigen. In comparative titrations of dourine sera with the two forms of antigen I have found that approximately one-tenth of the amount of serum necessary for a positive reaction with spleen antigen suffices for a clear positive reaction with trypanosome antigen. Very weak positive reactions with the former become clearly and strongly positive with the latter, which, therefore, should always be given the preference. The trypanosome suspension has also the great advantage of retaining a constant value for several weeks at least, for six to eight weeks if carefully prepared, and thus allows of the keeping of a uniform stock antigen.

What is the percentage of positive reactors in dourine outbreaks?—This of course varies according to the length of time the disease has been in existence in a stud or range herd before being checked by preventive measures. In the most extensive outbreak that we have had to deal with 456 positive reactors were found in a total of 2,000 animals tested; nearly 23 per cent. In an outbreak on an Indian Reservation, 127 animals gave positive reactions out of 1,464 tested, or less than 9 per cent. Usually it is between 15 and 20 per cent. Our experience indicates that 100 per cent of dourine infected animals, whether in active or latent stages of disease, give positive serum reactions, provided that an interval of two to three months has been allowed for an incubation period in the more or less resistant animals, less than one month being sufficient in most cases.

How does the value of the dourine test compare with the Wassermann test for syphilis?—The old name of horse syphilis still clings to dourine infections, especially among stock owners and the general public, and comparisons have been made both in regard to the nature of the disease and the diagnostic tests, tending to lead to mistaken conclusions.

The reaction in dourine by the method recommended in this paper is a specific one. A positive reaction in other diseases or with animals in which dourine infection could be excluded, remains unknown to us, while in every authentic case of dourine the reaction is invariably positive. In my whole experience there is only one case in dispute—a negative serum reaction being given where a symptomatic diagnosis of dourine was made. However, the symptomatic diagnosis may have been at fault; unfortunately, the animal was destroyed before any proof or disproof of dourine infection was forthcoming.

The very few cases on record where a negative dourine reaction at a first test was followed by a positive reaction at a second or later test can be accounted for by infection taking place only a few days before the serum was first collected, or by continued exposure to infection between the first and later test.

In syphilis, on the other hand, negative reactions are of more value for prognosis than for diagnosis. A positive reaction may become negative after a short course of treatment, returning again to positive if a cure is not effected. Further, it is admitted that a negative reaction is frequently given in primary syphilis and again at times in latent and tertiary syphilis. A source of error, operating in the negative

direction, is, as Noguchi has pointed out, in that human serum contains a variable amount of natural anti-sheep amboceptor, which in some cases may be sufficient to hide a positive reaction. Horse serum does not contain anti-sheep amboceptor, as I have found by many experiments, so that the anti-sheep haemolytic system can be used in horse serum tests with perfect reliance.

A positive Wassermann reaction may be given in several diseases in which syphilitic infection can be excluded, in leprosy, scarlatina, certain forms of tuberculosis and carcinoma. The Wassermann reaction is not specific. Owing to the great difficulty of obtaining a pure syphilitic antigen, the extract of a syphilitic liver was first used. Wassermann's original method. But, later on, it was found that non-specific extract of normal liver and other organs answered equally well, and such are now commonly used. The reaction in syphilis is not accordingly a true and specific antigen-antibody combination and is dependent upon more or less gross changes in the serum of syphilitic patients. It is not to be compared, therefore, and is greatly inferior to our test method for dourine either in delicacy, specificity or trustworthiness.

In conclusion, I venture to express absolute confidence in the complement fixation test for dourine as it is now presented, and to claim that apparent failures or discrepancies are due, not to the method itself, but to faulty technique on the part of the operators or of the collectors of the test serum.

APPENDIX No. 3.

EXPERIMENTAL DOURINE.

Horses.—*Tr. equiperdum* is always pathogenic for the horse. If introduced into the vaginal tract of a healthy mare it reproduces the disease in a similar fashion to that which follows an infective coitus. A subcutaneous inoculation of trypanosomes, obtained from a plaque, oedematous swelling or vaginal secretions, is followed in about 10 days time by a small swelling at the seat of inoculation. In the serous fluid of such a swelling multiplying trypanosomes are to be found. This primary lesion disappears in a few days and the disease evolves as in a case of natural infection, especially if the inoculation has been made via the genital organs.

It is essential that the material inoculated contains the specific trypanosome of dourine. Blood drawn from the general circulation of dourined horses rarely contains trypanosomes and thus rarely reproduces the disease when inoculated into healthy subjects. Direct transfusions of blood or inoculations of large quantities repeated at intervals over several weeks have failed to set up an infection. Only during a very active stage of the disease, as during a period of plaque eruption, is an inoculation of blood from the general circulation likely to be successful.

The virulence of a strain of dourine can be raised to a high degree by a series of rapid passages through young animals. In this way the disease is rendered more acute in foals or yearling horses, running its course in about six weeks in males and in about three months in females, a high and remittent fever being the dominant feature throughout (vide Chart No. II and compare with Charts Nos. I and III).

The trypanosome virulence for horses is raised to its maximum degree by passage through laboratory animals, viz., white mice, rats and guineapigs. In horses infected with trypanosome blood taken from a rat or guineapig the disease makes rapid and continuous progress. There is scarcely any remission of symptoms. Trypanosomes soon appear in the general circulation and are present there in varying numbers up to the time of death, which occurs in from six weeks to three months,

in adult horses. In this connection it is well worth noting that 11 horses and 1 donkey have been experimentally infected with trypanosomes which had been passed through a rat or guineapig, and that in all 12 animals trypanosomes appeared in the general circulation. On the other hand, 24 horses have been experimentally infected with trypanosomes of seven or eight different equine origins and previous to passage through laboratory animals, and in none of these 24 animals were trypanosomes ever to be seen in the general circulation. Similarly, hundreds of blood examinations for trypanosomes in cases of natural infection likewise proved negative.

This exalted trypanosome virulence and the acquired habit of the parasite to live in the bloodstream of horses after a passage through laboratory animals has been maintained for several generations in horses.

Laboratory animals.—Very great difficulty is experienced in transmitting equine dourine to laboratory animals. The white mouse seems to be the least resistant; even so, it may be necessary to inoculate series after series before obtaining a single successful infection. However, once this is accomplished, the trypanosome can be passed through rats, guineapigs, rabbits, dogs, cats, etc., with great ease, from an animal of one species to that of another and carried on indefinitely without loss of virulence.

The adaptation of the trypanosome for laboratory animals is not lost by subsequent passage through horses, at least not for several generations.

Mice and rats.—The transmission of Canadian dourine to laboratory animals was effected by a successful inoculation of a white mouse after hundreds of unsuccessful attempts had been made with dogs, rabbits, guineapigs, etc. The origin of the strain was a naturally infected mare. During a period of four weeks in which this mare presented fresh plaques almost every day, 60 white mice and 24 rats were inoculated with fluid collected from the plaques, rich in trypanosomes. The first 83 inoculations (59 in mice and 24 in rats) failed; the eighty-fourth (in the sixtieth mouse) succeeded. From this mouse others were infected without any difficulty and the strain was passed on through the usual laboratory animals. In mice and rats only one trypanosome cycle is represented. The parasites appear in the blood on the second or third day after inoculation and multiply with rapidity up to the time of death, which in mice occurs usually in three to five days, and in white rats in four to six days. Death takes place suddenly and with scarcely any warning symptoms. When rats are inoculated in batches of 15 to 25 at one time, as when preparing a trypanosome antigen for complement-fixation work, it may happen that 15 or more rats out of 20 will die within the space of two to three hours on the fourth or fifth day. Often there are one or two rats in every 25 that show more or less resistance. Twenty-three out of 25 rats inoculated may die on the fourth to fifth day, the twenty-fourth rat may live ten to fifteen days and the twenty-fifth rat twenty to thirty days notwithstanding the fact that the trypanosomes present in the blood of these two resistant rats become even more numerous than in the rats which die in the normal time of four to six days. It would appear that in a mouse or rat infection when the end of the first trypanosome cycle is reached there is a sudden release and absorption of toxin which causes sudden death in the great majority of cases. Not more than 5 to 10 per cent of infected rats survive the shock, and these succumb to the second or third trypanosome cycle.

Guineapigs.—The duration of infection averages between six and eight weeks, as a rule. However, with a highly virulent rat strain of dourine guineapigs may succumb within two to four weeks. The appearance of trypanosomes in the blood is remittent or intermittent and the parasites are never as numerous as in rat's blood. Few symptoms can be noted and death occurs with suddenness. After a number of generations in guineapigs the strain is apt to lose virulence but this is easily restored by passing through a series of rats.

Rabbits.—Infected rabbits present a train of more or less characteristic symptoms, viz., oedematous swellings of the external genital organs and of the ears, purulent conjunctivitis and other ocular lesions, cutaneous lesions, progressive emaciation, cachexia and death in from one to three months. The parasites appear in the general circulation with remissions or intermissions and are rarely numerous.

Dogs.—Pronounced and typical symptoms of trypanosomiasis are presented: fever, oedemas of the skin and genital organs and very severe ocular troubles; the eyeball protrudes and has an anxious, staring expression (exophthalmia), and there is a keratitis and staphyloma. There is locomotory disturbance and a great feebleness and a tendency to sleep almost continuously. Emaciation and paralytic conditions precede death.

As already stated, in the first direct transmission of dourine to laboratory animals all attempts to infect dogs, rabbits, and guineapigs failed, and success was only achieved through a white mouse. The trypanosome was then passed through rats and no further difficulty was experienced in infecting other species of small animals. Further, when a horse was infected with a dourine strain maintained in laboratory animals, the trypanosomes recovered in the blood of this horse were always infective and highly virulent for dogs and all other species of laboratory animals. So easy had transmission then become that dogs were infected by injection, by eating the flesh of horses which had succumbed to trypanosome infection.

A horse was destroyed *in extremis* and a post-mortem examination was made immediately after death. Trypanosomes were found present in the blood of all the organs and muscles examined. A piece of flesh was fed to two dogs with little expectation that they would become infected. However, three weeks after the feeding the dogs appeared to be easily fatigued, dull and somewhat depressed. On the twenty-ninth day a careful search of blood taken from the ear revealed a very few trypanosomes. On the thirty-fifth day the parasites were fairly numerous, and the dogs appeared to be very ill. The eyes had a bulging, stony appearance. The face was rather puffy and expressionless. For several days there had been attacks of vomiting and diarrhea. For short intervals the animals were very restless, but most of the time were very depressed and slept for many hours at a stretch.

An intravenous injection of 0.2 grammes of Salvarsan (606) in 100 c.c. of alkaline solution was given and within forty-eight hours all trypanosomes had disappeared from the blood stream. The change and improvement were astonishing. At the time of the injection the animals were so weak that they had to be propped up and supported. Three days later they were running about, barking and playing. However, on the fifty-ninth day in one, and on the sixty-fourth day in the other, there occurred a relapse. Symptoms recommenced and the parasites reappeared in the blood. A second injection of Salvarsan, 0.25 grammes in the one and 0.3 grammes in the other, was given. This time there was scarcely any noticeable change. The parasites remained in the circulation, the symptoms progressed and death occurred in eighty-one days in the one case and in ninety-five days in the other.

TABLE III.—Showing the exalted virulence of *Trypanosoma equiperdum* for horses after passage of the parasite through white rats and guinea (pigs).

Animal.	Inoculation of trypanosomes before passage.	Duration of disease and recovery.	Inoculation of trypanosomes after passage.	Predominating symptom.	Duration of disease and death.	Remarks.
Mare (41) aged	Feb. 17, 1907	(See Chart II) 5½ years	June 12, 1912	Remittent fever	145 days	Trypanosomes in blood stream.
Foal (36).....	Aug. 16, 1908	4½ years	Feb. 14, 1913	"	29 days	"
Foal (37).....	Aug. 26, 1908	4½ years	June 12, 1912	"	90 days	"
Foal (45).....	Jan. 8, 1909	3½ years	"	"	84 days	"
Stallion (30) (15 year old)			Feb. 14, 1913 (intravenous)	"	45 days	"
Mare (90) (2 year old)			Mar. 30, 1915 (intravaginal)	"	86 days	"
Gelding (130) (8 year old)			Feb., 23 1917 (subcutaneous)	"	81 days	"
Donkey (183) 7 year old			June 7, 1915 (subcutaneous)	"	117 days	"

APPENDIX No. 4.

TRYPANOSOMA EQUIPERDUM.

The microscopical appearance of the trypanosome of dourine is very similar to that of other pathogenic trypanosomes of the *Brucei* type. In morphology, vitality and general biological features, there is little to distinguish it from the trypanosomes of surra, nagana, mal de caderas and sleeping sickness of man. Very common features of the dourine trypanosome are (1) the presence of a vacuole bordering upon the centrosome and (2) the blunted, cut-off or fish-mouth appearance of the posterior extremity.

Tr. equiperdum does not require any insect intermediary carrier or host and completes its life cycle in the body of its natural host—the horse—and is directly communicable from one horse to another.

Tr. equiperdum is the only known pathogenic trypanosome of tropical or subtropical origin that is maintained by natural means in the temperate zones and in countries far removed from tropical conditions.

In its natural host, naturally infected, *Tr. equiperdum* does not live as a true blood parasite but rather as a tissue parasite, selecting special organs, mucous membranes and the skin for its development and life cycle. In these respects the trypanosome of dourine differs from the trypanosomes of surra, nagana and the other trypanosome diseases.

The appearance, development, multiplication, conglomeration or agglutination, phagocytosis and intracellular digestion of *Tr. equiperdum* takes place in a single oedematous patch or plaque, and the whole phenomenon can be traced within, and usually occupies, 3 days. The following is an example:—

In a young mare, experimentally infected with a trypanosome strain (before passage through laboratory animals), three- to four-day periods of fever recurred at twenty-four to twenty-eight day intervals and, at the same time or preceding the rise in temperature by one to two days, an oedematous swelling of the labium pudendi. Examinations of the fluid contents of the patch were made on the first sign of tumefaction or oedema and continued at intervals of a few hours throughout the stages of eruption and absorption. The procedure at each examination was to make a fine needle puncture and with the drop of serous fluid exuding make two dry smears and one wet coverglass-celled preparation. The latter was examined immediately, the former being stained by the Giemsa method. In the first specimens thus obtained the trypanosome could be located only with great difficulty being so few in number. In the subsequent specimens increasing numbers and multiplication forms were noted up to the thirty-sixth hour. At the 40th hour conglomerations of trypanosomes and attachment to the phagocytes were noted. At the forty-fourth hour nearly all the trypanosomes were ingested by the macrophage cells and in various stages of digestion and disintegration. At the forty-eighth hour no trypanosomes could be identified as such, though in some of the macrophage cells fragmenting parasitic nuclei could be made out. On the third day the swelling diminished in size, was further reduced on the fourth and was quite absorbed by the end of the fifth day. No trypanosomes could be seen in the specimens taken on these days.

Similar observations were made on a number of occasions and in different horses. In one case in which depigmentation of the skin of the labia pudendi was taking place, it was noted that simultaneously with the macrophagic ingestion of the parasites numbers of these phagocytic cells were crowded with blackish granular pigment. In smear preparations many of these swollen cells ruptured and let escape masses of black granules.

Agglutination and phagocytosis of the trypanosomes cannot be observed in all cases and, in fact, only in the minority. The later history of these cases shows that the infection was well tolerated and the inference is that agglutination and phagocytosis is an indication of increasing resistance and of the process of immunity.

Little is known of the nature and properties of trypanosome toxins. In the acute, generalized infections of small laboratory animals, and in which only one trypanosome cycle is represented, death apparently results from the sudden release and rapid absorption of toxin at the time of maximal trypanosome multiplication and commencing disintegration. In the chronic, intermittent disease in the larger animals commencing in a local infection, the trypanosome repeats its cycle again and again and the toxic action on the nervous system appears to be progressive and cumulative.

Attempts to cultivate *Tr. equiperdum* on artificial culture media and to isolate a toxin have not succeeded in the hands of the writer.

APPENDIX No. 5.

REGULATIONS RELATING TO MALADIE DU COÏT.

By Order in Council dated 22nd July, 1911, in virtue of the Animal Contagious Diseases Act, R.S.C., 1906.

1. No animal which is affected, or suspected of being affected with Maladie du Coït shall be permitted to run at large or to come in contact with any animal which is not so affected, and no such animal shall, in any case, be used for breeding purposes.

2. Any inspector may declare to be an infected place within the meaning of "The Animal Contagious Diseases Act," any common, field, stable, or other place or premises where animals are found which are affected or suspected of being affected with Maladie du Coït.

3. No horse, ass or mule shall be removed out of any place so declared to be an infected place without a license signed by an inspector.

4. The Veterinary Director General may from time to time order the slaughter, castration or other disposition of animals affected with Maladie du Coït.

5. Inspectors are hereby authorized to inspect any animals affected with Maladie du Coït, or suspected of being so affected or which have been in contact with animal so affected, or suspected of being so affected or which have been in any way whatever exposed to the infection of Maladie du Coït, and may order any such animals to be collected, detained, isolated, castrated, or otherwise dealt with as may to them appear advisable.

6. The expenses of, and incidental to the collection of, isolation, seizure, castration, or otherwise dealing with animals for the purposes of these regulations, shall be borne by the owners of the animals, and no indemnity shall be allowed to the owner in case of damage arising out of or resulting from such actions, except as herein-after provided.

7. No entire horse, ass or mule nor any ridgling more than one year old shall be permitted to run at large on unfenced lands in the province of Alberta or in that portion of the province of Saskatchewan lying west of the third principal meridian.

8. Any entire horse, ass or mule or any ridgling more than one year old found running at large within the area defined above may be seized and held on the order

of any duly authorized veterinary inspector of the Department of Agriculture, shall forthwith whenever possible notify the owner of the said animal of such seizure, and the said animal, if not claimed within thirty days of such seizure, may be castrated, and no indemnity shall be allowed to the owner in case of damage arising out of or resulting from said castration, seizure or detention.

9. Animals affected with *Maladie du Coit*, may on an order signed by a duly appointed veterinary inspector acting under special instructions from the Veterinary Director General, be forthwith slaughtered, and the carcasses disposed of as in such order provided, and compensation may be paid to the owners of such animals if and when the Act so provides.

10. Before an order is made for the payment of compensation in any of the cases aforesaid there must be produced to the Minister of Agriculture a satisfactory report, order for slaughter, and certification of valuation and slaughter, all signed by an inspector.

APPENDIX No. 6.

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 'Dourine, its pathogenicity and, a test of the efficacy of drug treatment...', Jour. Comp. Path. & Ther., 1911.
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 and HAWKEN, S., - 'Trypanosomes found in Canadian Mammals', Parasitology, Vol. V, 1912.
 GUTHRIE, M. V. 'Dourine', Ann. Rep. V.D.G., 1911. Appendix No. 10.
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EXPLANATION OF PLATES

Plates I, II and III

Natural infection of Stallion No. 35, showing eye lesions, fluctuating oedema, progressive emaciation and final stages of the disease

Plate IV

Stallion, No. 33, naturally infected with Dourine (1905-1907)

Plate V

Experimental infection of mare No. 66, showing a ring-shaped or hollow-centre "plaque," the ninth to appear, in the tenth month inoculation. Figure 1.

Fig. 2. - Natural infection of mare No. 42, showing facial paralysis

Fig. 3. Paraplegia in a naturally infected mare.

Plate VI

Figs. 1, 2 and 3. Experimental infection in young fillies, showing typical oedematous patches of the anus (fig. 1), of the labia pudendi (fig. 2), and of the skin between the buttocks. (Fig. 3).

Figs. 4 and 5. - Natural infection of mare No. 159, showing depigmental skin of the vulva and anus and the transient character of such depigmentation. Fig 5 shows the condition ten months later than fig. 4.

At the time these photographs were taken (figs. 1-5), trypanosomes were present in the oedematous fluids, in each case.

Plate VII

Trypanosoma equiperdum in the oedematous fluid of a swollen labium pudendi, showing the development, multiplication, agglutination, phagocytosis and digestion of the parasites- all taking place within the space of 48 hours.

Plate VIII

Trypanosoma equiperdum

Plate IX

Trypanosoma equiperdum the flagellated micro-organism causing Dourine, as seen in the vaginal mucus fluid (figs. 1-8) and in the oedematous fluid from swollen patches of the vaginal membranes (figs. 10-18); taken from a naturally infected mare (animal No. 36)

EXPLANATION OF CHARTS

HISTORY CHARTS NOS. I, II, AND III

These charts epitomize the history of forty-two cases of dourine in horses, twenty-two being naturally infected and twenty experimentally inoculated.

The chief points of interest and conclusions are:—

The fatality in stallions.

The tolerance and recovery in mares and the relative immunity obtained to reinfection by natural means.

Tolerance and acquired immunity to inoculations of a natural strain of dourine (horse) is broken by inoculations of a laboratory strain (rats and guineapigs) and death follows.

In some of these mares the period of observation is fifteen years, in others twelve, ten, and a lesser number of years. Mares spontaneously cured of the disease have shown their usefulness for work purposes and for breeding. No case of natural transmission to the offspring has been observed. The four healthy stallions kept for breeding these naturally cured mares escaped infection.

Specific serum properties, as shown by the complement-fixation test, are retained in some cases for many years after recovery and cure; in others these properties are lost in about five years (see Appendix 2).

History Chart No. II shows the origin and genealogy of a strain of dourine in horses, and the exalted virulence obtained by serial passage through foals, and is supplemented by: Temperature Charts Nos. I, Ia, Ib, Ic; II, IIa, IIb; III; IV; IX and X.



PLATE I.

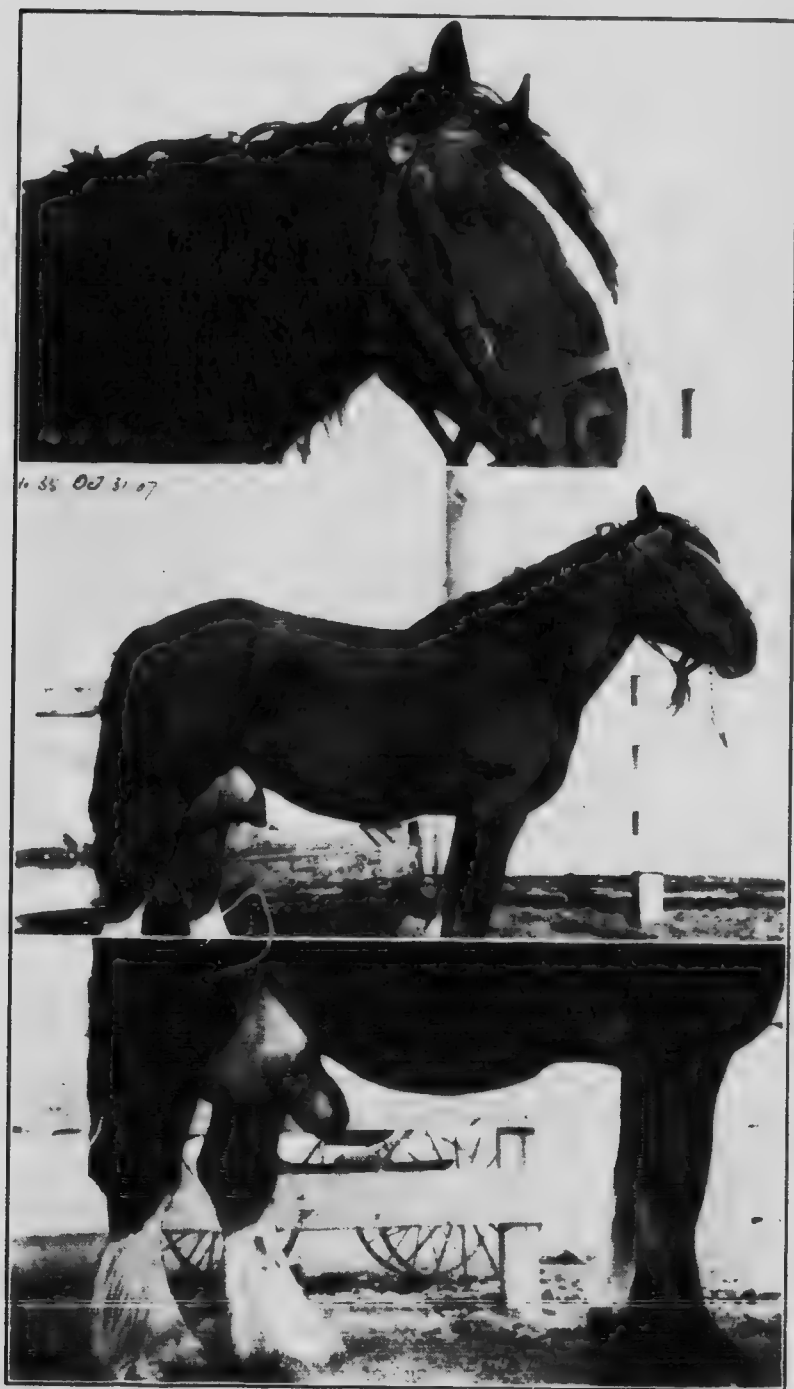
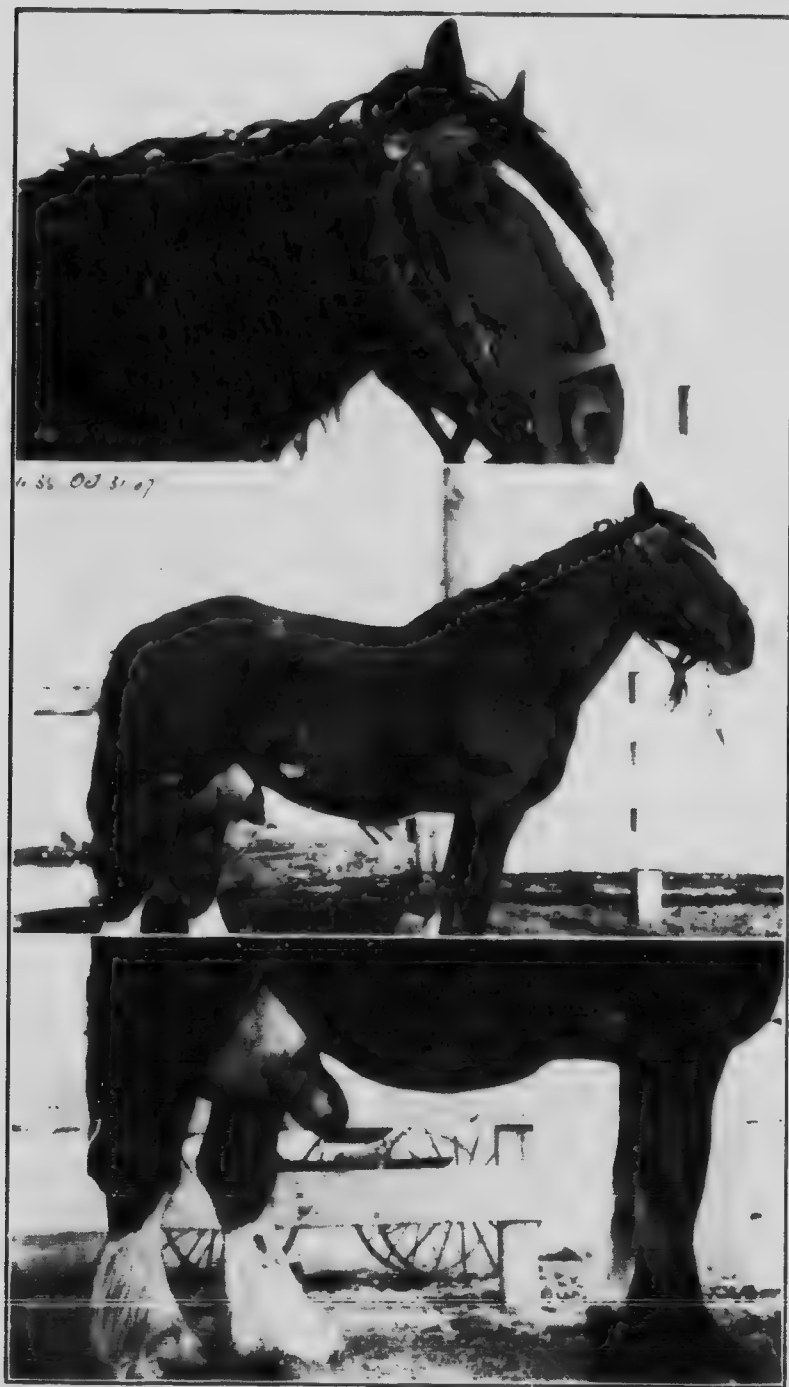




PLATE I.



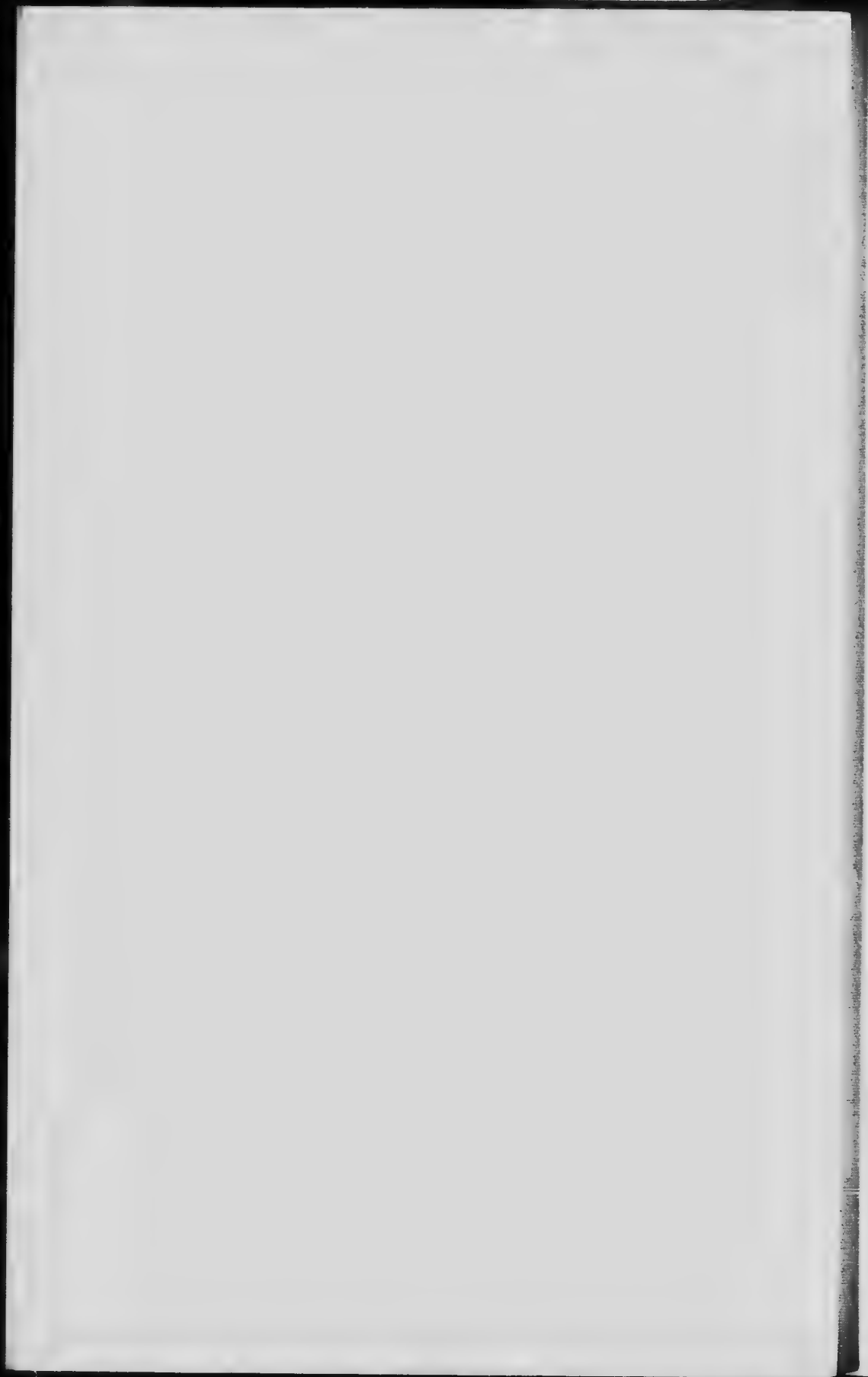


PLATE II.

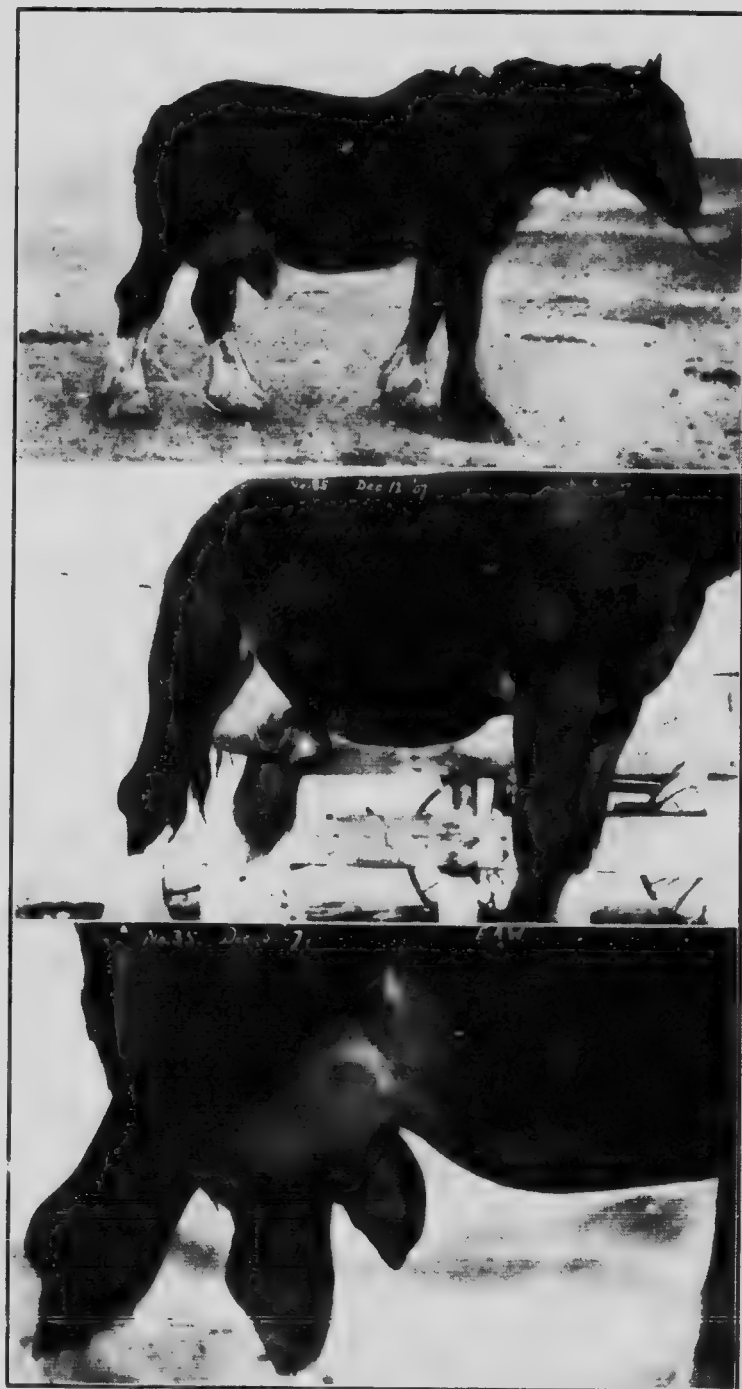




PLATE IV.



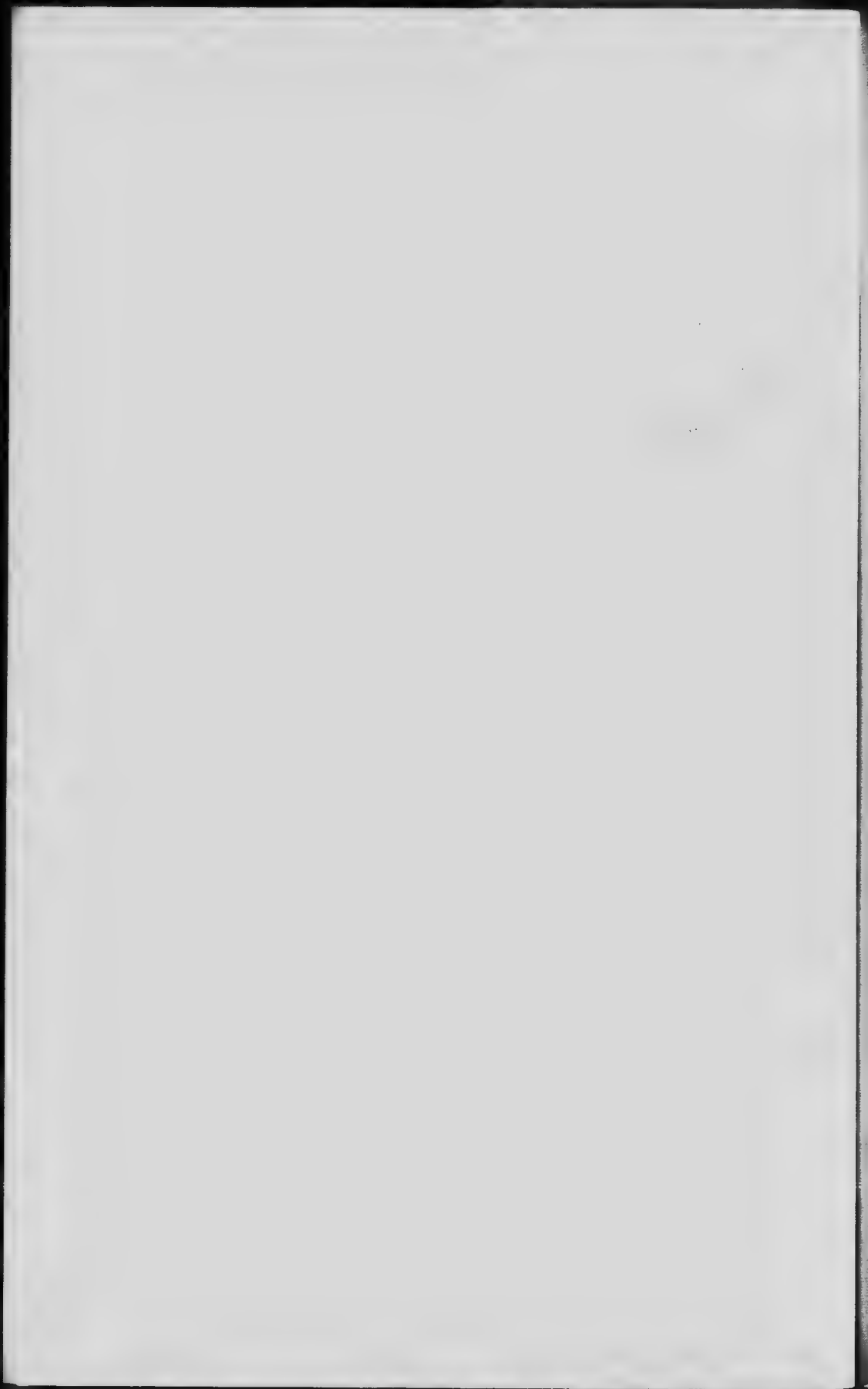


PLATE V.



FIG. 1.



FIG. 2.



FIG. 3.





FIG. 1.



FIG. 2.



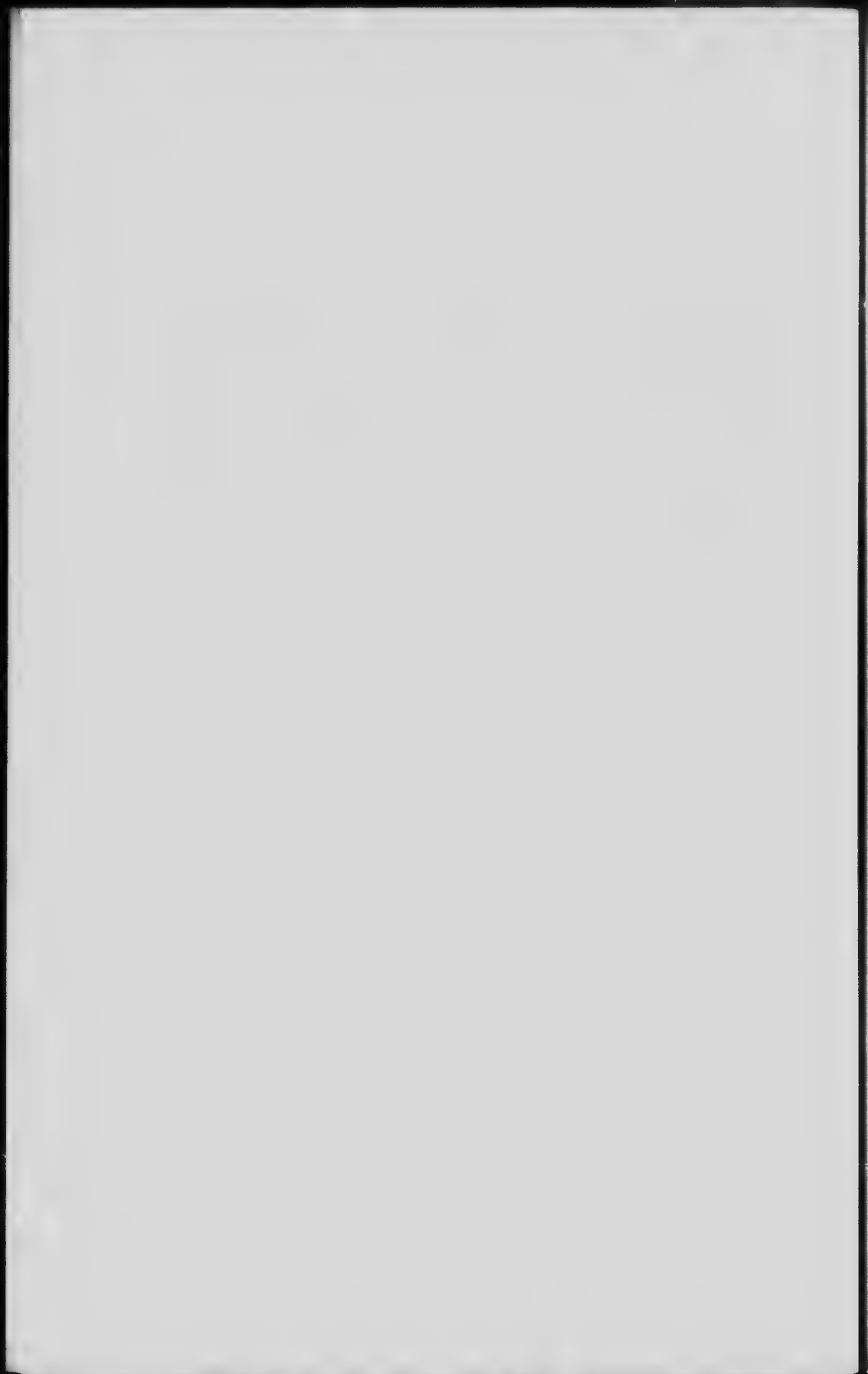
FIG. 3.



FIG. 4.

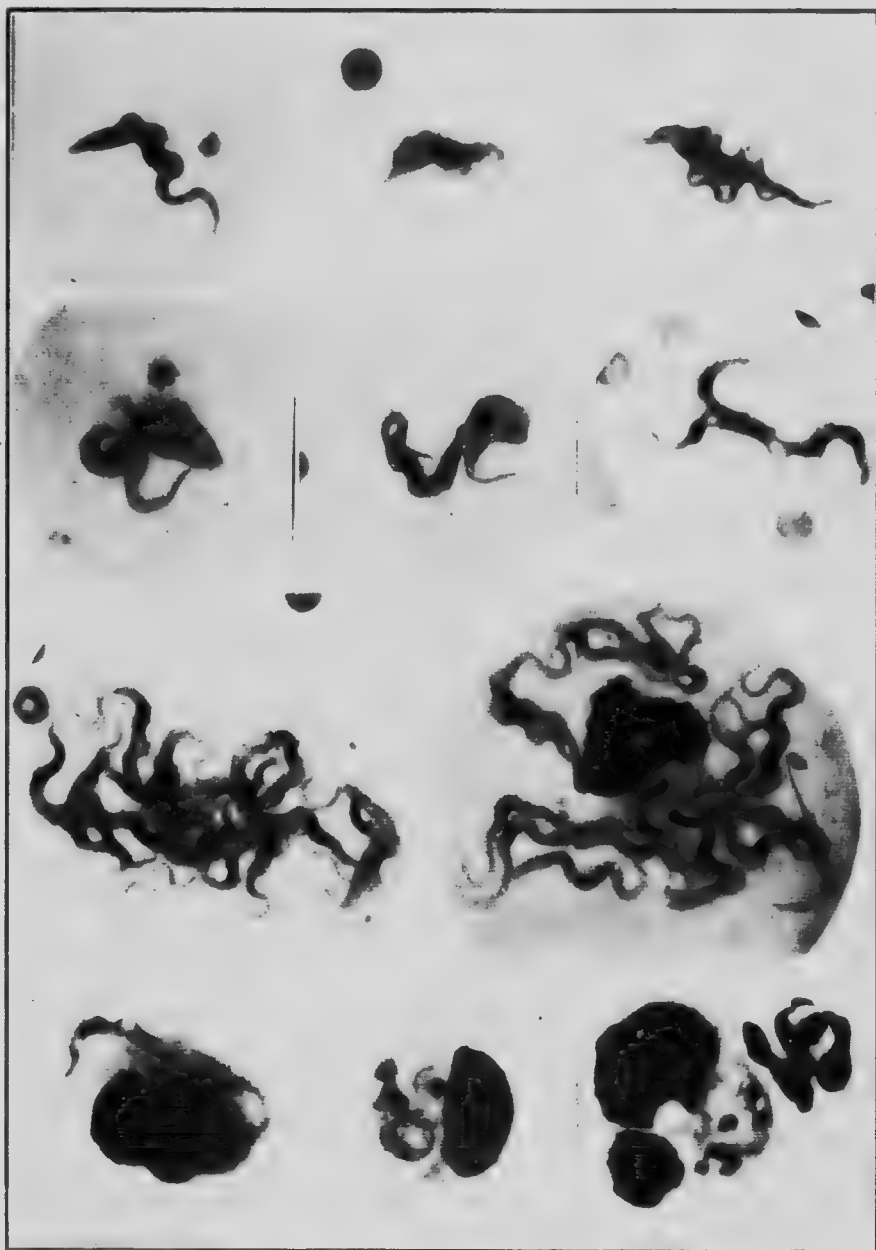


FIG. 5.



Trypanosoma equiperdum.

PLATE VII.



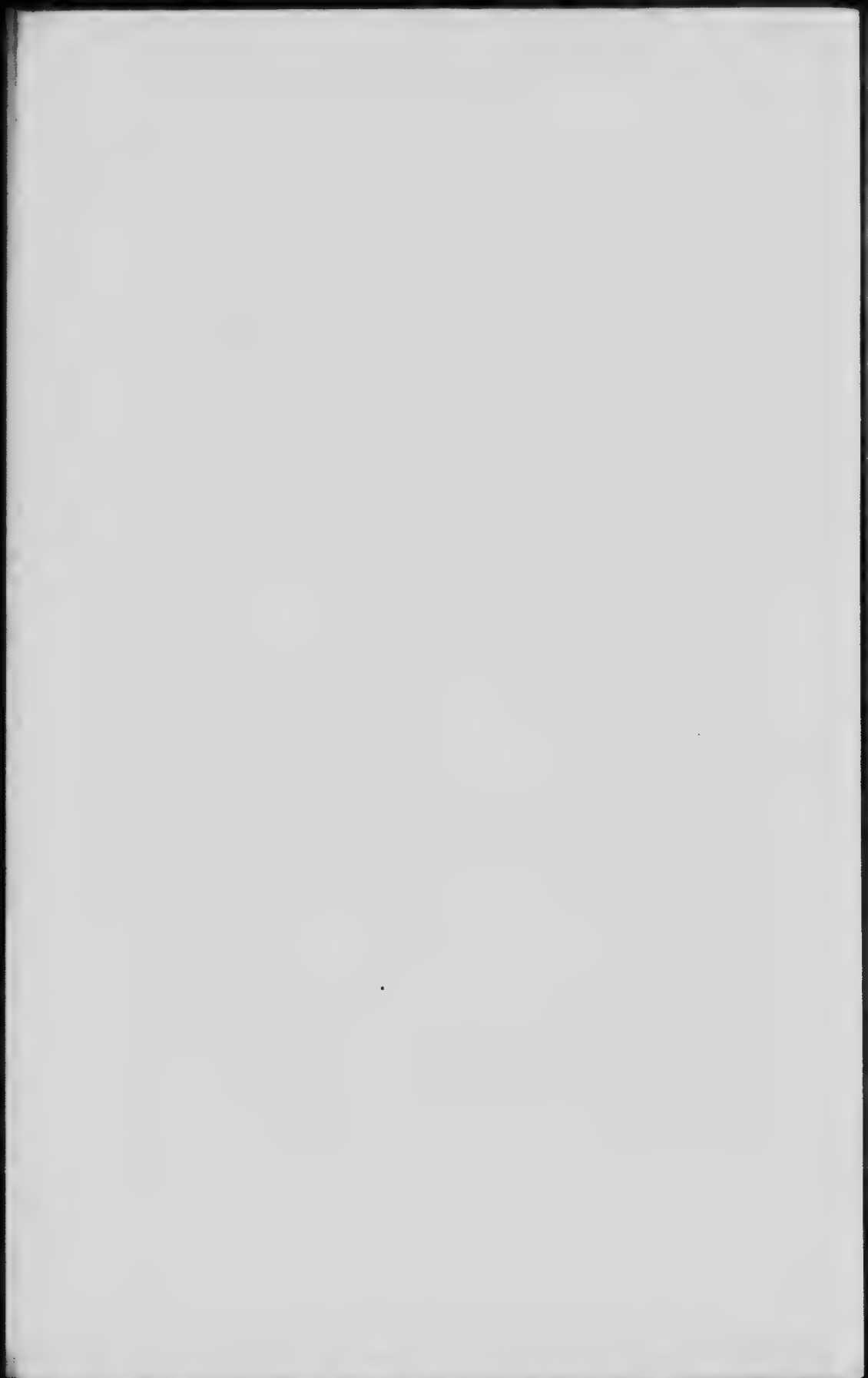


PLATE VIII



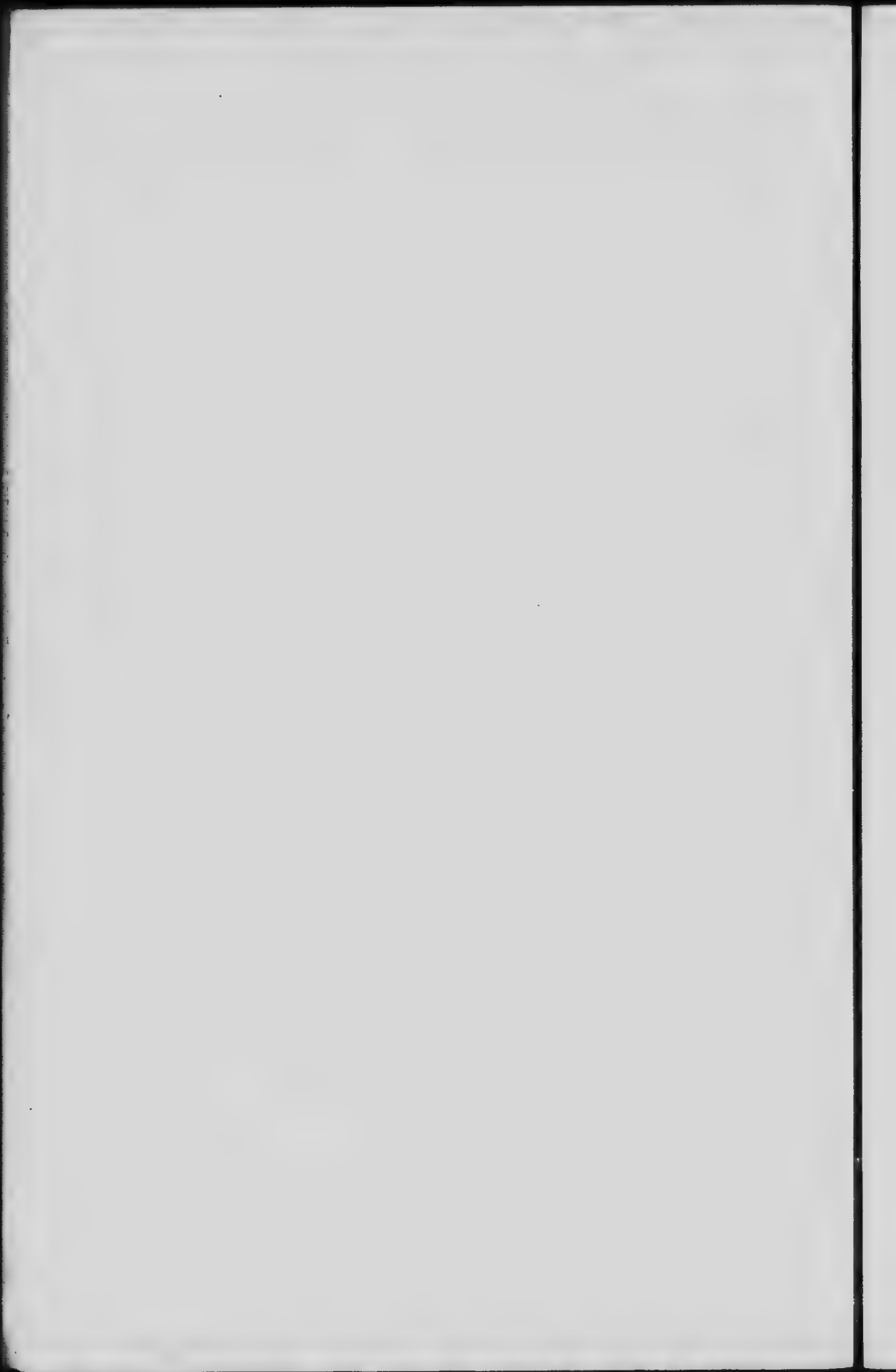
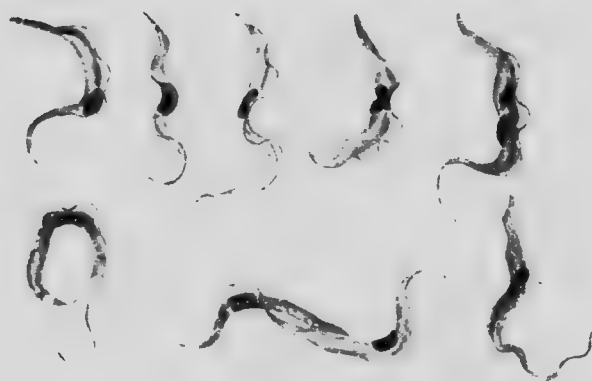
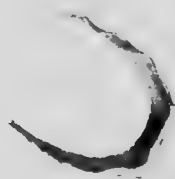
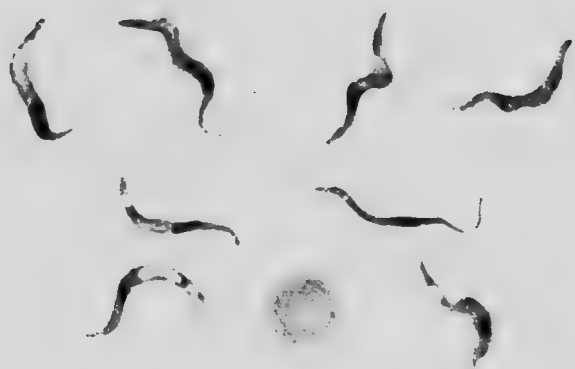


PLATE IX

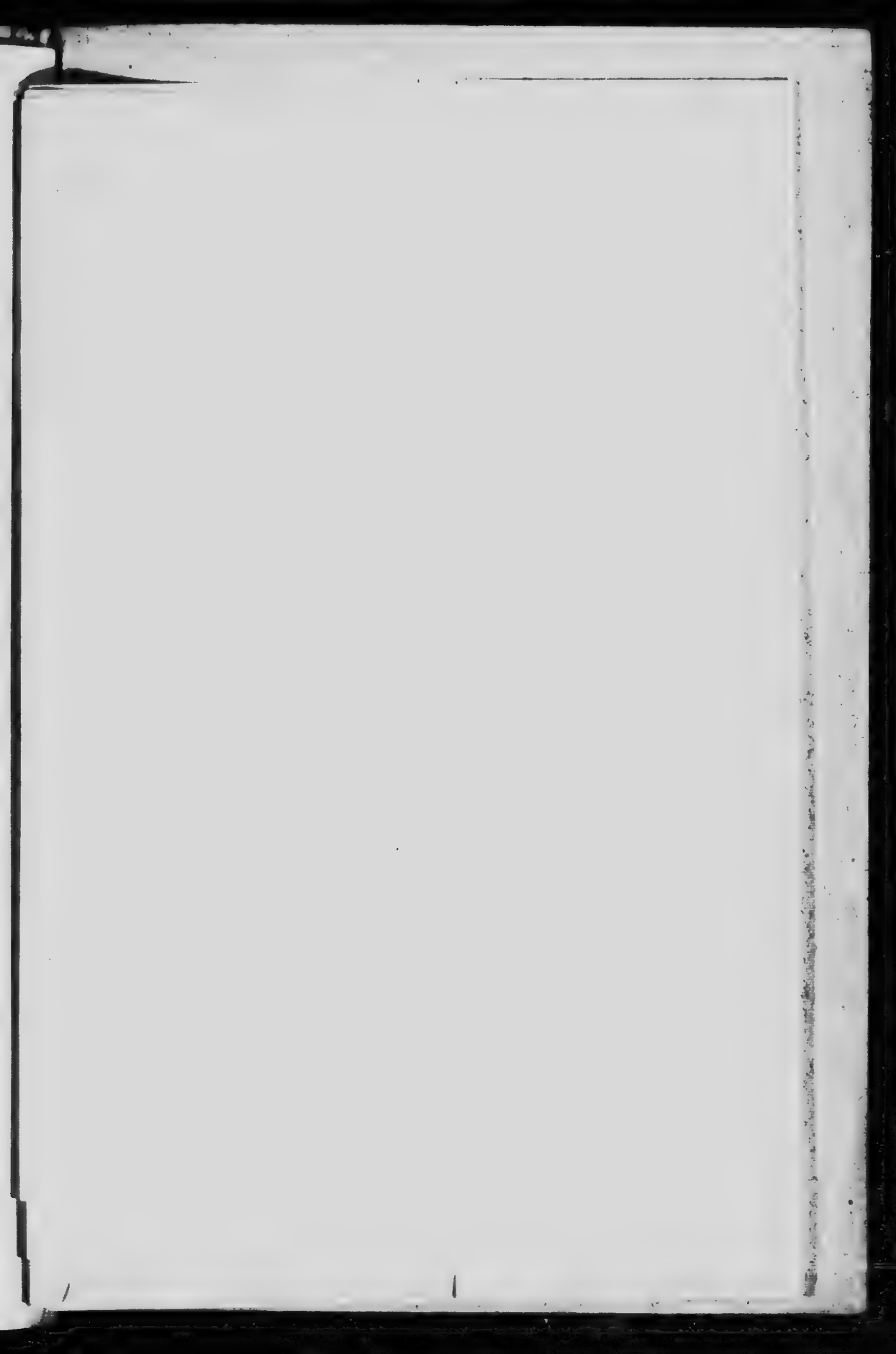


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HISTORY-CHART N

HEALTH OF ANIMALS BRANCH.—VETERINARY RESEARCH

	1905	1906	1907	1908	1909	1910	1911	1912
Stallion (31)	Natural infection.	Oedema Destroyed.						
Stallion (33)		Oedema, anaemia, emaciation, paralysis, death (July 24)						
Stallion (72)	Natural infection.	Oedema, Cachexia, death (Oct 24)						
Mare (4)	Natural infection.	Tolerant. Covered by Stl. 31.	Normal. Covered by Stl. 72.	Normal. Bred to healthy stallion.	Foaled			Foaled.
Mare (5)		Tolerant.	Normal.	Normal.	Normal.	Normal.	Normal.	Inoculated with trypanosomes from mare 165
	Natural infection	3 coverings by Stls. 31 and 33.	3 coverings by Stl. 72.	(Employed as a work mare)				
Mare (19)		Tolerant. 5 coverings by Stls. 31 and 33.	Normal. 3 coverings by Stl. 72.	(Employed as fast driving mare for 6 years, and as a brood				
Mare (7)	Natural infection	Tolerant	Inoc. of trypanosomes from mare 36.	Normal.	Normal. Inoc. of trypanosomes from foal 6f.	Normal.	Normal.	Accidentally hurt. Destroyed
Mare (9)	Natural infection	Tolerant. 7 coverings by Stls. 31 and 33.	Normal. Inoc. of trypanosomes from mare 36.	Recurring oedema of vaginal mucosa.	Normal.	(Death from colic.)		
Mare (13)	Natural infection	Tolerant. 1 covering by Stl. 31.	Normal. Bred to healthy stallion.	Normal. Foaled.	Inoc. of trypanosomes from Foal 12f.	A period of high fever after inoculation.	Normal.	Normal. C.F.
Mare (17)	Natural infection.	Tolerant. 1 covering by Stl. 33.	Twice inoculated, trypanosomes from mares 9 and 29	Normal.	Twice inoculated, trypanosomes from foals 1f, 7f.	Normal.	Normal.	Normal.
Mare (22)	Natural infection.	Tolerant. Ovaries removed.	(Normal health and condition maintained.)					Inoc. of trypanosomes from mare 165. Oedema. C.F. + Inoc. trypanosomes from Mare 165. Decem
Mare (23)		(Apparently recovered)	Very good condition.	No symptoms)				Inoc. of trypanosomes from mare 165. No reaction C.F. + Inoc. trypanosomes from mare 165. Infection. Death
Mare (24)	Natural infection	Tolerant. 7 coverings by Stls. 31 and 33.	Normal. Foaled	Slight Oedema.	Normal. Foaled	Normal.	Foaled.	C.F. Foaled
Mare (25)	Natural infection.	Vaginal discharge, emaciation, paraplegia.	Slow improvement	Inco-ordination. Further improve-	(General health and condition good, though inco-ordination of hind limbs remains as a chronic symptom.)			C.F. + Inoc. trypanosomes from a rat. Death

trypanosomes from foal 11f

Death
51 days

HART No. 1.

RESEARCH LABORATORY, LETHBRIDGE, ALBERTA.

1912	1913	1914	1915	1916	1917	1918	1919
foaled.		CF. ++	CF. +++ Foaled Failing health	Death (Jan 25)			Complement Fixation positive
culated with try- panosomes in mare 185	CF. +++ Oedema, trypano- some periods	CF. +++ Failing health	CF. +++ Death (Mar 15)				Complement Fixation positive
and as a brood mare for 4 years	CF. -	CF. -					Complement Fixation negative
						Died foaling	

mal.	CF. +++	CF. +++	CF. +++	CF. +++	CF. +++	CF. +++	CF. +++	Complement Fixation positive
work mare	Foaled.	Foaled	Foaled		Foaled			
mal.	CF. +	CF. +						Complement Fixation positive
	Foaled	Died foaling						
of ano- from 185. ma.	CF. +++ No symp- toms from March to December.	CF. +++ Inoc. of trypano- somes from inf. rat. Death.						Complement Fixation positive. Feb. 3 1914 Inoc. of trypanosomes from an infected rat May, 10 1914 -Death.
of on- from 185 tion	CF. +++ Inoc. of trypano- somes from inf. rat Death.							May 5 Inoc. of trypano- somes from an infected rat Aug. 4 1913 -Death. Complement fixation positive
	CF. - Foaled.	CF. -	CF. - Foaled.	CF. -	Foaled		Foaled	Complement Fixation negative
gh a	CF. +++ Inoc. of trypano- somes from a rat. Death.							Compliment Fixation positive May, 5 1913 Inoc. of trypanosomes from an infected rat. Sept., 29 1913 -Death

HISTORY-CHAR HEALTH OF ANIMALS BRANCH--VETERINARY RE

Serial generation and passage of <i>Trypanosoma equiperdum</i>	1906	1907	1908	1909	1910	1911	1912
1st. Stallion (35). Natural infection		Oedema, koratosis, anaemia, emaciation, paraplegia.	Death				
2nd. Mare (36). Natural infection		Oedema vaginal m m, uterine paraplegia, 7 plaques.		Paralysis.	Death.		
3rd. Foal (39). Experimental inoculation, trypanosomes from mare 36		Oedema, paralysis	Death				
3rd. Mare (41). Experimental inoculation, trypanosomes from mare 36		Slight oedema vaginal m m.	29 plaques	Normal.	Normal	Normal	
3rd. Foal (29). Experimental inoculation, trypanosomes from mare 36		Slight oedema vaginal m m	14 plaques.	Normal.	Normal	Raised healthy foal.	Normal C.F.
4th. Filly (60). Experimental inoculation, trypanosomes from foal 29			Recurring oedema of labia pudendi	Normal.	Normal.	Normal.	
4th. Foal (31). Experimental inoculation, trypanosomes from foal 29	See Temperature Charts I, Ia, Ib, Ic.		Oedema, fever paroxysms, ocular and paraplegic symptoms		Recovering	Absence of symptoms.	
4th. Foal (51). Experimental inoculation, trypanosomes from foal 29.	See Temperature Charts II, IIa, IIb.		Recurring oedema l p., fever paroxysms, 3 plaques, paraplegic symptoms		No symptoms Apparent recovery		
5th. Foal (11). Experimental inoculation trypanosomes from foal 51	See Temperature Chart III		Recurring oedema; fever paroxysms	Slight paraplegic symptoms	Apparent recovery.		
6th. Foal (6). Experimental inoculation, trypanosomes from foal 11	See Temperature Chart IV		Recurring oedema, remittent fever, debility		Death		
7th. Foal (7). Experimental inoculation, trypanosomes from foal 61			Recurring oedema, remittent fever, urticaria		No symptoms Apparent recovery after 3 injections of arsenophen nylglycin.		
8th. Foal (8). Experimental inoculation, trypanosomes from foal 71				Fever Death 41 days.			
9th. Foal (11). Experimental inoculation, trypanosomes from foal 81	See Temperature Chart IX			Fever Death 51 days.			
10th. Foal (12). Experimental inoculation, trypanosomes from foal 111	See Temperature Chart X			Fever Death 51 days			

Y-CHART No. 2.

NARY RESEARCH LABORATORY, LETHBRIDGE, ALBERTA.

1912 1913 1914 1915 1916 1917 1918 1919

									June 12, 1912. Inoc. dourine trypanosomes from guinea-pig. November 4, 1912. Death. (See Table IV.)
nal.	C.F. ++	C.F. ++ Fouled.	C.F. +	C.F. +- Fouled	C.F. - +	C.F. - +	C.F. - + Fouled.	Complement Fixation,—Weak positive reactions.	
nal.		C.F. —	C.F. — Fouled	C.F. — Fouled.				Complement Fixation,—negative	
nce oms.								Feb. 14, 1913. Inoc. dourine trypanosomes from an infected rat. Mar. 15, 1913. Death. (See Table IV.)	
								June 12, 1912. Inoc. dourine trypanosomes from an infected guinea-pig. Sept. 10, 1912. Death. (See Table IV.)	
								June 12, 1912. Inoc. dourine trypanosomes from an infected guinea-pig. Sept. 1, 1912. Death. (See Table IV.)	
	C.F. —	C.F. — Fouled.					Fouled.	Complement Fixation,—negative.	

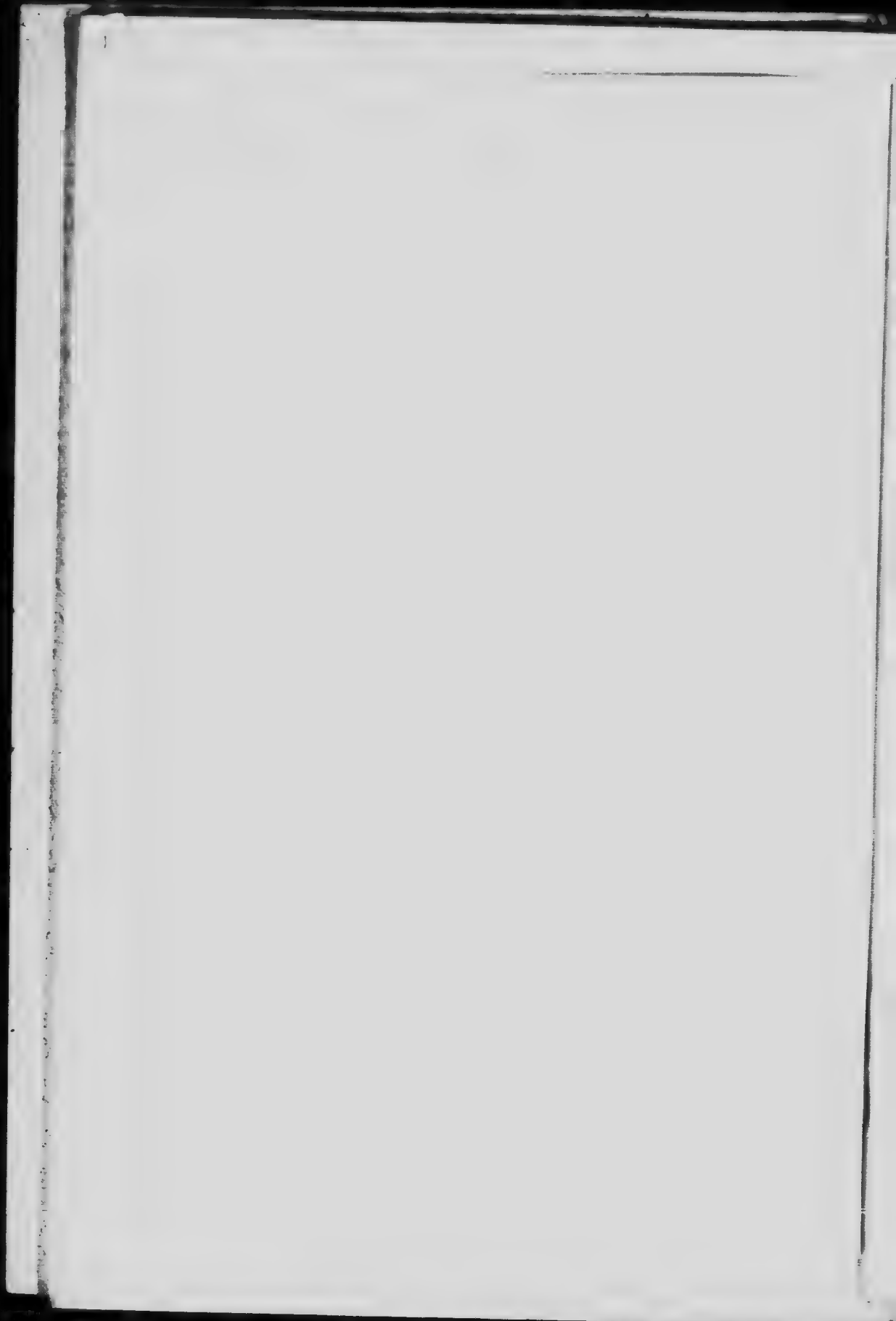
HISTORY-CHART HEALTH OF ANIMALS BRANCH - VETERINARY RESEARCH

	1906	1907	1908	1909	1910	1911	1912	
Mare (26) (2 yr. old). Experimental inoculations of blood from stallion 33. Nov. 21 to Dec. 3, 1906.		Absence of definite symptoms	1 plaque Aug. 22. Trypanosomes detected	Normal	Foaled			Foaled
Gelding (43) (2 yr. old). Experimental transfusion of 600 c.c. of blood from mare 36. Apl. 24, 1907.		Intermittent paraplegic symptoms	Normal	Normal	An excellent riding horse			Died of influenza
Mare (66) (3 yr. old). 4 experimental inoculations of trypanosomes from mares 29 and 41 and from foal 71. Apl. 21, 1908. May 21, 1909.			Oedema of lab. pudendi. 8 plaques. Trypanosomes present	1 plaque	Normal	Made a good saddle mare for 4 years		
Mare (67) (3 yr. old). 3 experimental inoculations of trypanosomes from mares 29, 41 and 82. Feb. 3, Aug. 27 '08.				Oedematos swellings under abdomen	Normal	Foaled		
Mare (69) (3 yr. old). 1 experimental inoculation of trypanosomes from mare 29. Aug. 27, 1908.			Recurring oedema of labia pudendi, rich in trypanosomes	Normal recovery	Normal recovery	Made a good saddle mare for 4 years		
Mare (70) (2 yr. old). 2 experimental inoculations of trypanosomes from mares 29 and 41. Oct. 4, 1907.		Recurring oedemas, rich in trypanosomes	Normal recovery	Foaled		Foaled		
Mare (71) (3 yr. old). Experimental inoculation of 100 c.c. of blood from mare 29. Aug. 29, 1908.			Absence of definite symptoms	Normal	Foaled	Foaled		
Mare (78) (5 yr. old). Experimental inoculations after recovery from a natural infection from stallion 33.		Tolerant. Slight oedema of vaginal mucosa.	Normal. Inoc. of trypanosomes from foal 3f.	Normal. Inoc. of trypanosomes from foal 4f.	Normal	Employed as a fast driving mare for 5 years		C.F.
Mare (52) (5 yr. old). Natural infection by stallion 33.		Tolerant. Slight oedema and paraplegic symptoms.	Normal	Employed as a saddle mare for 10 years				
Mare (71) (7 yr. old). Natural infection			Oedemas of vaginal mucosa and of labia pudendi, rich in trypanosomes	Normal recovery	Normal health maintained			C.F.
Mare (82) (7 yr. old). Natural infection			Recurring oedema of vaginal mucosa, labia pudendi and under abdomen. Trypanosomes frequently detected	Normal recovery	Normal health			
Mare (80) (5 yr. old). Natural infection (1913) by stallion 182								
Mare (84) (4 yr. old). Natural infection (1913) by stallion 182								
Stallion (182) (3 yr. old). Natural infection (1913)								Plaques and Trypanosomes present

RY-CHART No. 3.

ARY RESEARCH LABORATORY, LETHBRIDGE, ALBERTA.

1912	1913	1914	1915	1916	1917	1918	1919
	Fouled	CF - Fouled	CF Fouled Ill health	Died			Complement Fixation, negative
Died, from influenza							
for 4 years	CF. +++ Ill health Paralysis Death (16 Sept)						Complement Fixation, positive Relapse of disease and death after 4 years of toler- ance and normal health
	CF - Fouled	CF - Fouled		Fouled		Destroyed (Dec)	Complement Fixation, negative
Idle mate for 4 years	CF -	CF - Fouled		Fouled			Complement Fixation, negative
	CF - Fouled	CF - Fouled		Fouled			Complement Fixation, negative
	CF -	CF -				Normal	Complement Fixation, negative
CF -	CF -	CF -					Complement Fixation, negative Immunity acquired
mate for 5 years		Fouled	Fouled	Died in foaling.		Destroyed (Dec)	Complement Fixation, negative
	CF -	CF -					Complement Fixation, positive
CF -	CF -						Complement Fixation, positive
Destroyed							Complement Fixation, positive
	CF -	CF -					Complement Fixation, positive
	CF. +++ Plaques Inoc- ordination	CF. ++ Fouled (Mule)	CF. +++ Fair condition	CF. +++ Paraplegic symptoms, Emaciation	Died		Complement Fixation, positive
	CF. +++ Paraplegic symptoms Died (Aug. 15)	CF. +++					Complement Fixation, positive
Plaques and oedema. Trypan osomes present	CF. +++ Castrated.	CF. +++	CF. +++ Worked.	CF. +++ Failing health.	CF. +++ Decline and death		Complement Fixation, positive



T. Chart I.

Score-foot,
(No. 3. f.)
Age 6 weeks

1908

Mo	August	September
Day	1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31	1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30
106		
105		
104		
103		





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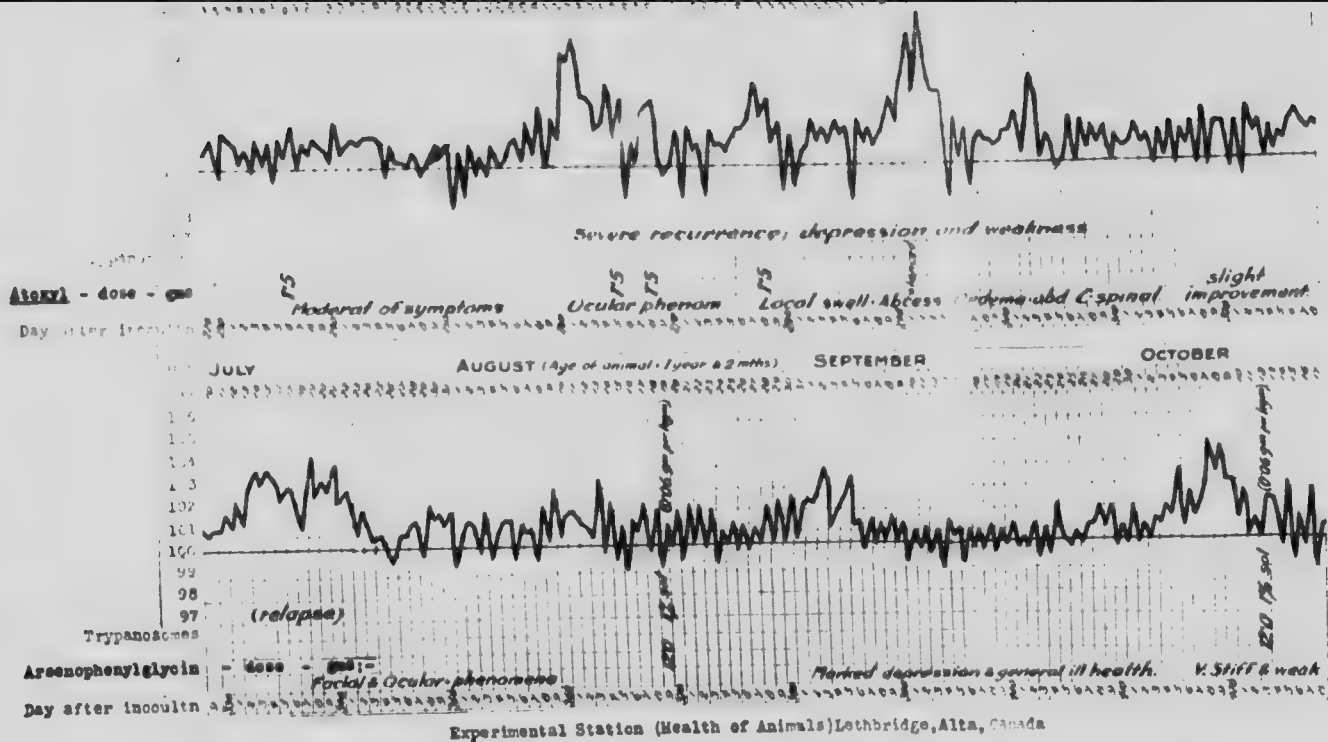
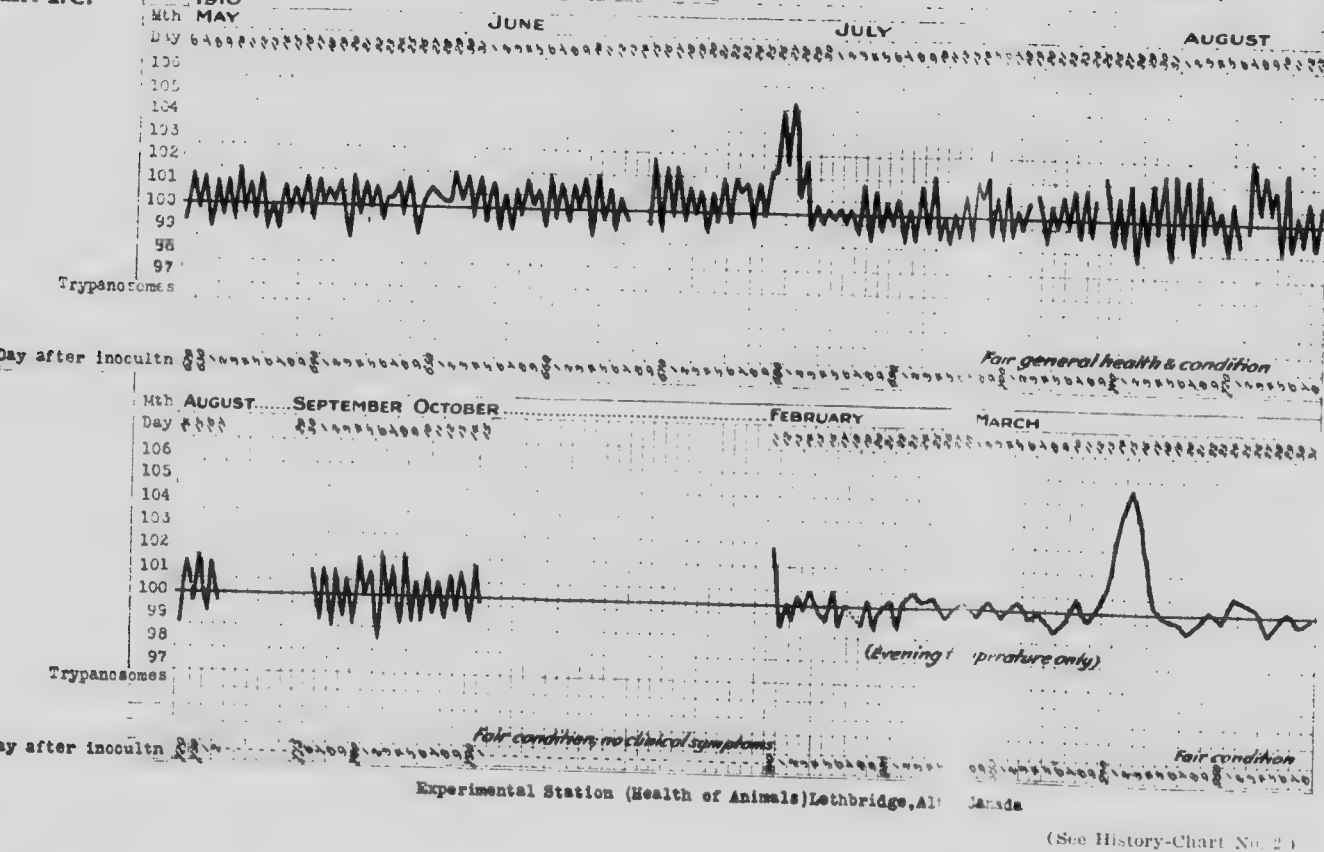


Chart I.b.

Horse, No. 3. f.

Experimental Dourine.

(E. A. Watson.)

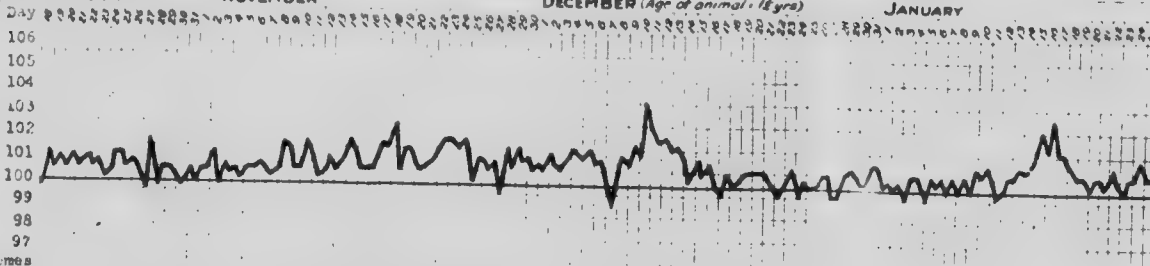
Continued: 1909

Mth OCTOBER

NOVEMBER

DECEMBER (Age of animal: 18 yrs)

1910 JANUARY



Trypanosomes

Little change or slight improvement in general health and condition.

Day after inoculn

1910

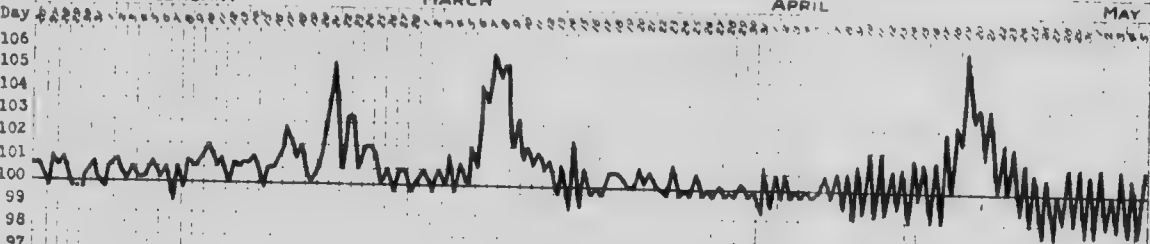
Mth JANUARY

FEBRUARY

MARCH

APRIL

MAY



Trypanosomes

Marked facial and ocular symptoms

Day after inoculn

Experimental Station (Health of Animals) Lethbridge, Alta, Canada

Chart I.c.

Experimental Dourine.

(E. A. Watson.)

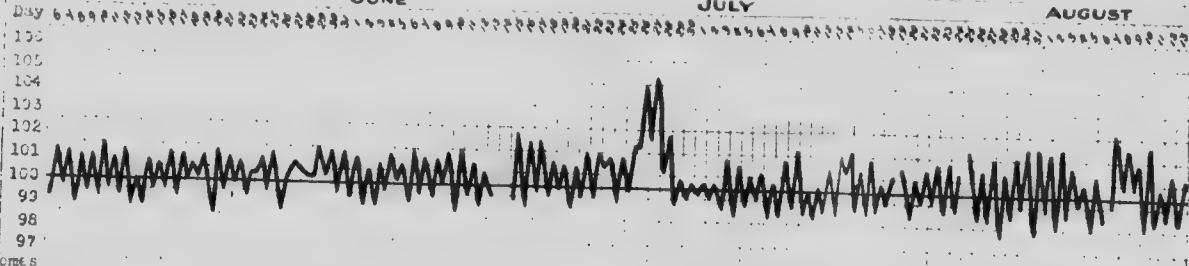
1910

Mth MAY

JUNE

JULY

AUGUST



Trypanosomes

Fair general health & condition

Day after inoculn

1910

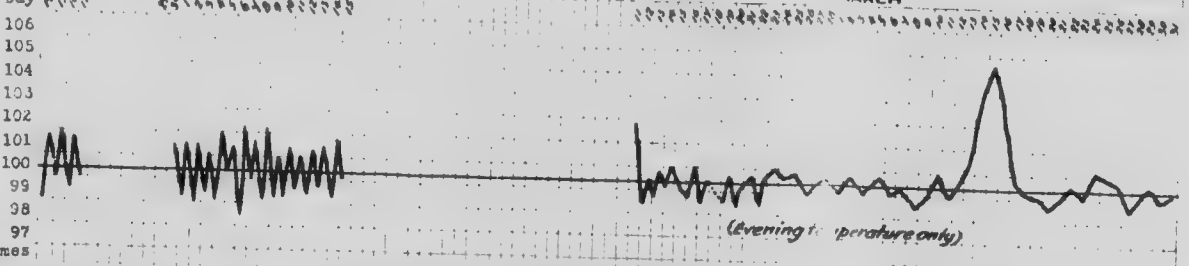
Mth AUGUST

SEPTEMBER

OCTOBER

FEBRUARY

MARCH



Trypanosomes

*(Evening temperature only)**Fair condition; no clinical symptoms*

Day after inoculn

Fair condition

Experimental Station (Health of Animals) Lethbridge, Alta, Canada

(See History-Chart No. 1)

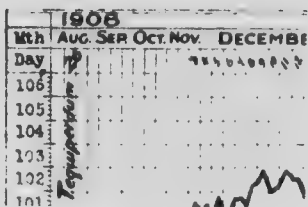


T. Chart II.

Horse.

(No. 5. f.,
filly.)

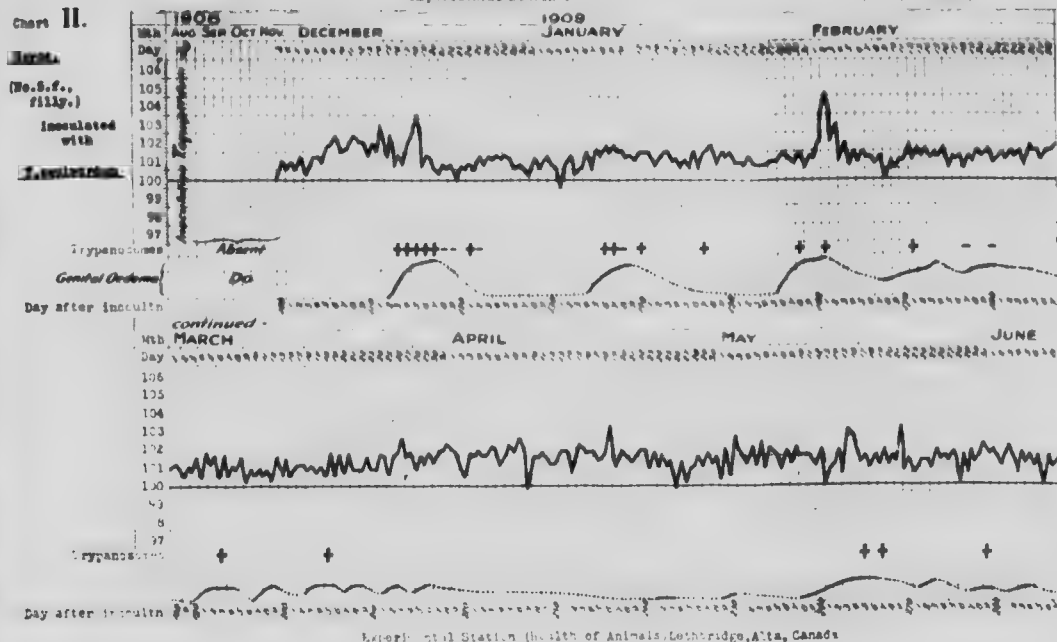
Inoculated
with



T. Chart II.

Experimental Design.

(E. A. Watson.)



Part IIa

Continued-1909

Норве

No. 5.f.

(*Untreated*)

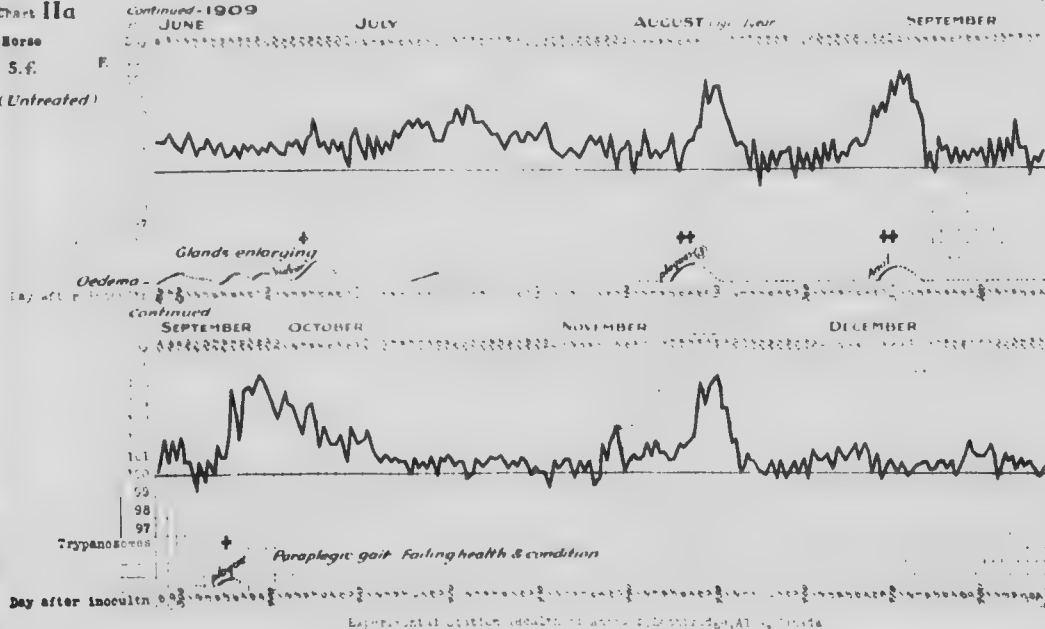
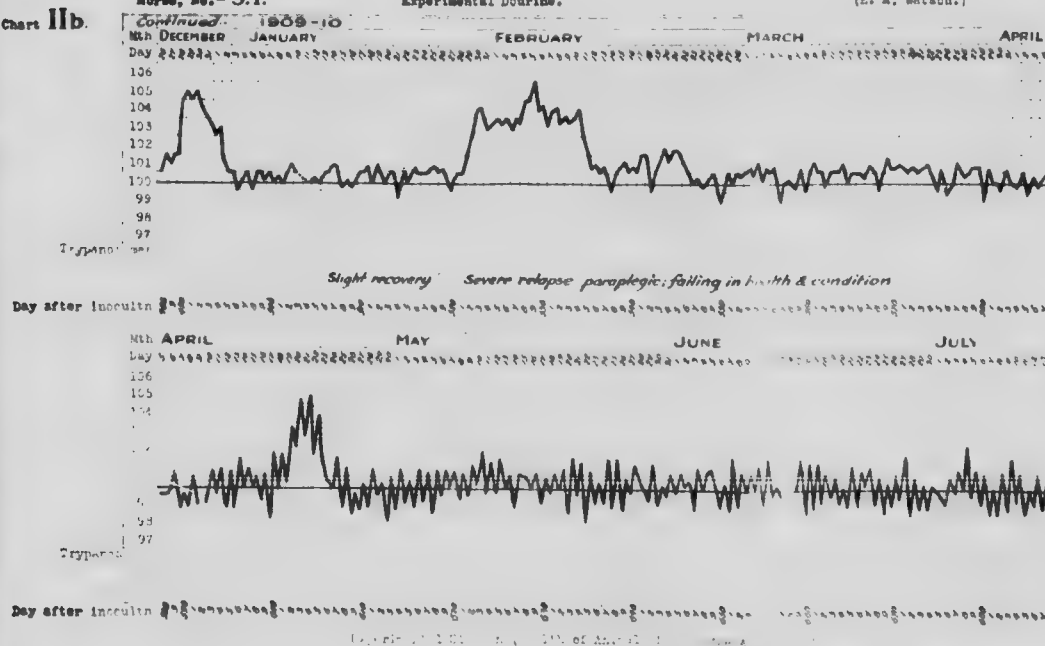


Chart IIb.

Korea, No. - 5.f.

Experimental Dourine.

(E. A. Watson.)



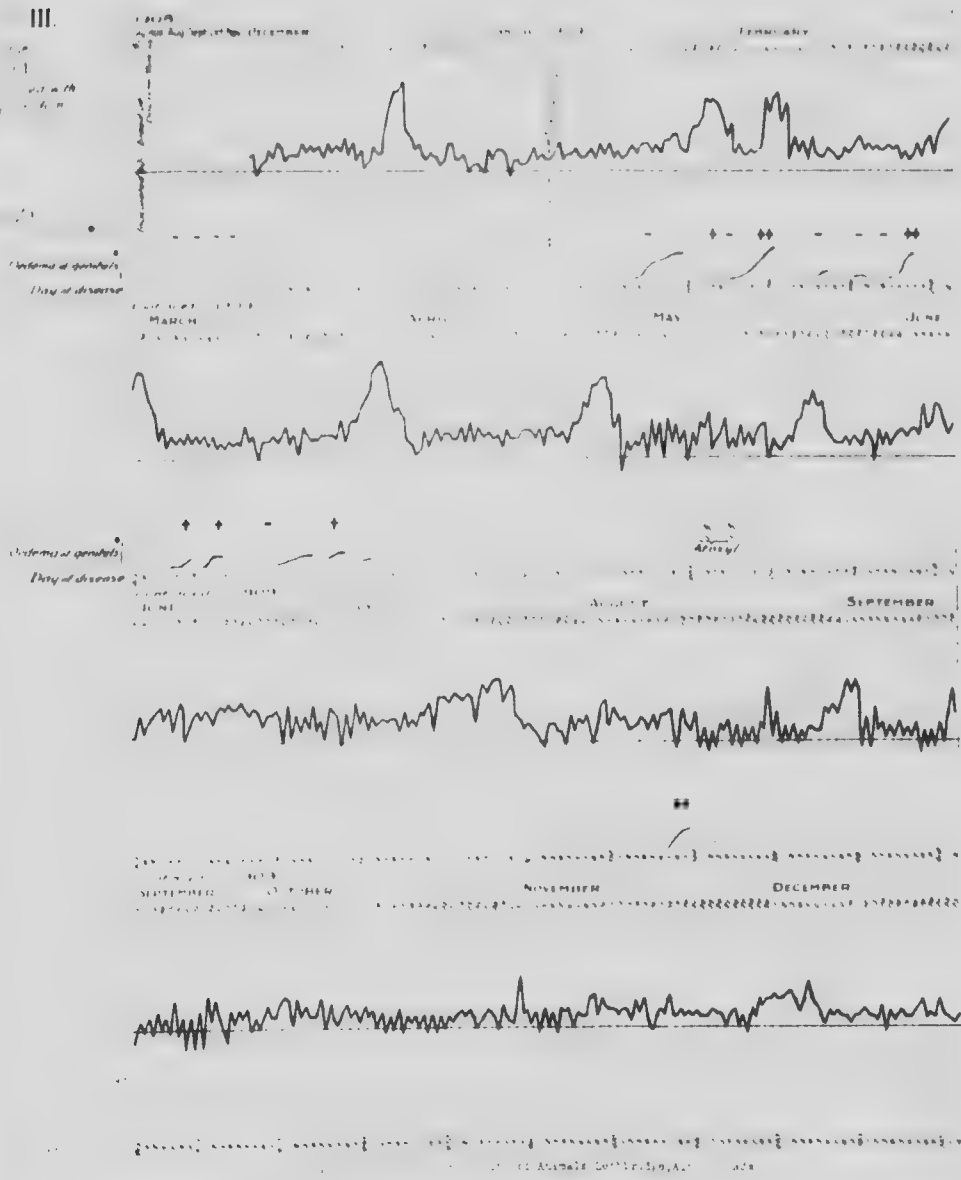
T.

For
Ta

$\frac{1}{2} \cdot p$
 $\frac{1}{2} \cdot m$

T III.

Horse No. 1
 1st inoculation
 2nd inoculation

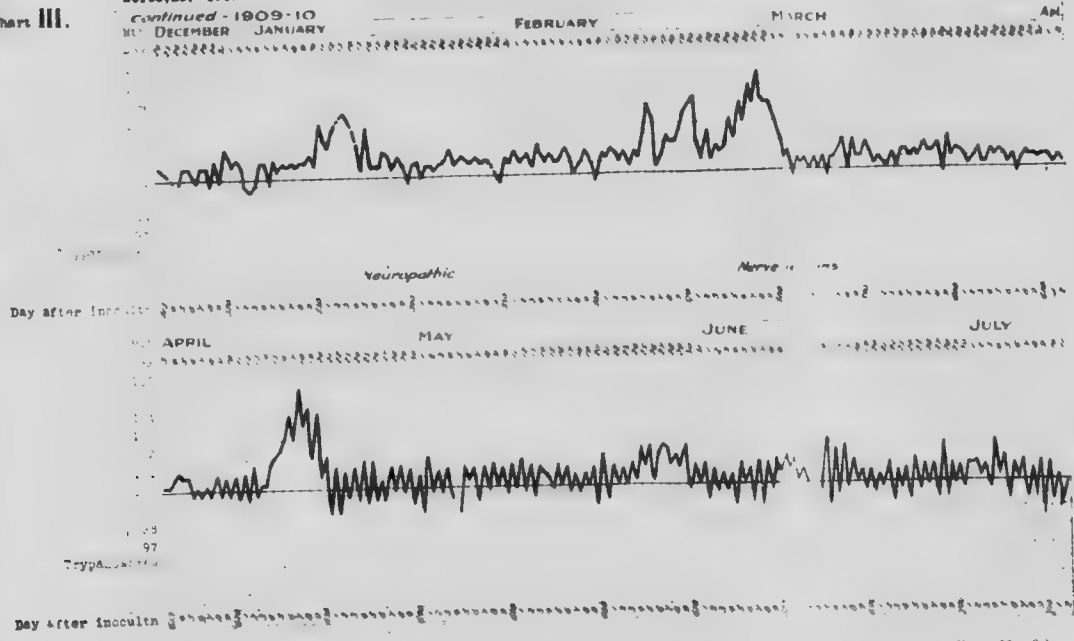


T. Chart III.

Horse No. 1
 continued - 1909-10
 1st DECEMBER JANUARY

Experimental Results

(P. A. Wilson)



T
T
T

Tr
(a
b

T IV.

Horse

No 6

Filly Age 6 mths

contaminated with
T. purpurum

+ present
- absent

Day of Disease

1909 continued
JUNE



77

(a
b

IV.
Horse
No. 6
Age 6 mths
Treated with
arsenic

+ present
- absent

Day of Disease

1909 Continued
JUNE

JULY

AUGUST

Animal 1 year old SEPTEMBER

Treated by

a- 0.05 g. per kg.
b- 0.06

Injections of
Arsenophenylglycol

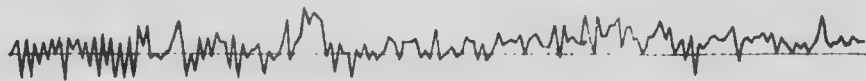
Day of Disease

1909 Continued
SEPTEMBER

OCTOBER

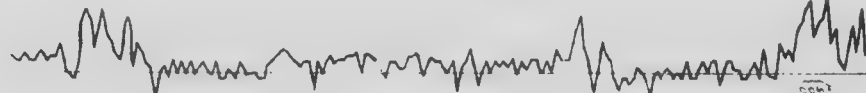
NOVEMBER

DECEMBER



General depression. Weak in hind limbs and joints. Iron & Arsenic given in feed daily. Some improvement apparent.

DECEMBER JANUARY FEBRUARY MARCH



Depression, weak sluggish action, very unhealthy condition.

(Combined treatment)
Arsenophenylglycol
Sodium arsenite

IV
continued

1910
MARCH APRIL

MAY

JUNE

JULY



More active and better nourished

Dullness and debility

JULY AUGUST SEPTEMBER OCTOBER



General debility, weakness & emaciation

X DEATH

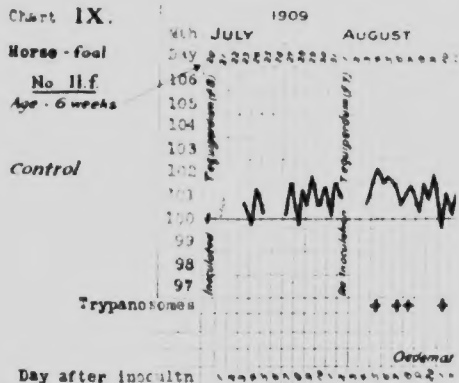
12 Years duration

Key

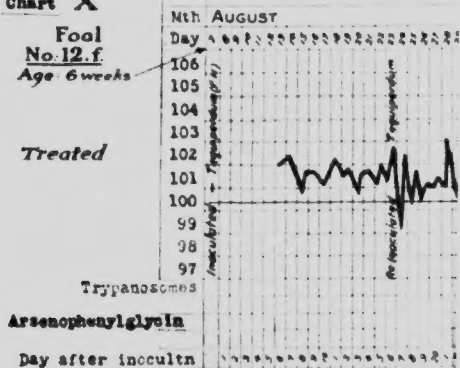
T.

T

T. Chart IX.



T Chart X



T. Chart IX.

Horse - foal
No. 11.f
Age - 6 weeks

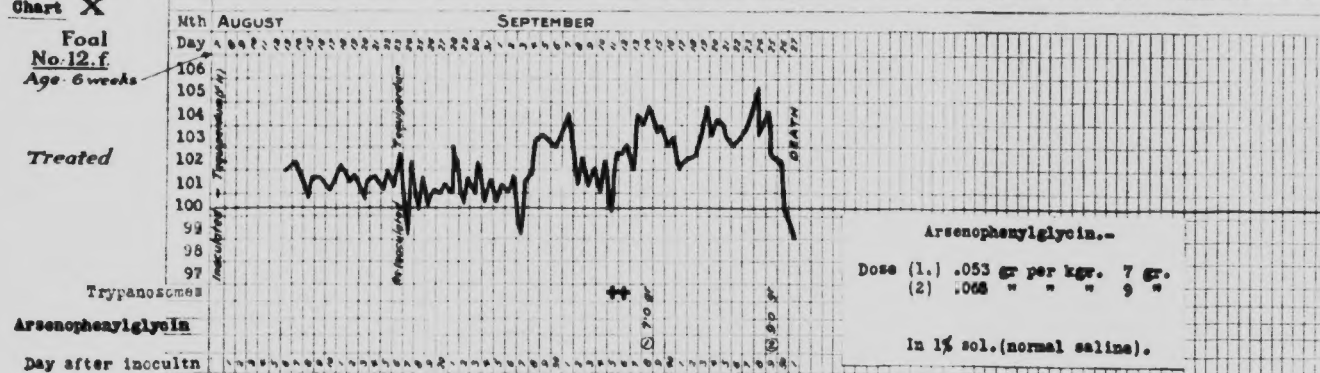
Control



T Chart X

Foal
No. 12.f
Age - 6 weeks

Treated



Experimental Station (Health of Animals) Lethbridge, Alta, Canada



